



THE JOURNAL OF  
ENDOCRINOLOGY



# THE JOURNAL OF ENDOCRINOLOGY

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VOLUME 4

1944-46

CAMBRIDGE  
AT THE UNIVERSITY PRESS

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JOHNSON REPRINT CORPORATION  
111 Fifth Avenue, New York, N. Y. 10003

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# THE ARTIFICIAL INDUCTION OF LACTATION IN THE BOVINE BY THE SUBCUTANEOUS IMPLANTATION OF SYNTHETIC OESTROGEN TABLETS

BY S. J. FOLLEY AND F. H. MALPRESS, *From the National Institute for Research in Dairying, University of Reading*

(Received 17 December 1943)

The important role played by oestrogens in mammary development has been established by numerous experiments on small animals [for review see Folley, 1940]. Similar results for the ruminant were reported by de Fremery [1936, 1938] who caused mammary growth in virgin goats by inunction of the udder site with ointment containing oestradiol monobenzoate; lactation was subsequently induced in these animals, but only after further treatment with anterior-pituitary extract containing prolactin. This work agreed with current views according to which the effects of oestrogens on lactation, both potential and established, were regarded as purely inhibitory. The adequacy of this theory was first seriously called in question by the experiments of Folley, Scott Watson & Bottomley [1940, 1941a], who showed that copious lactation could be produced in virgin goats by inunction of the udder region with ointment containing diethylstilboestrol. No anterior-pituitary extract was necessary, and lactation was initiated and the yield increased to a maximum of about 1.5 l. daily during the period of oestrogen administration. After stopping treatment the yield declined at a rate characteristic of a normal lactation cycle. Two nulliparous heifers treated in a similar way failed to respond to the same extent, giving only a very small yield of colostrum fluid [Folley *et al.* 1941b]. Lewis & Turner [1941] confirmed these results for the goat, causing abundant lactation by the subcutaneous injection of diethylstilboestrol in oil. The same authors have more recently reported similar successes in this species following administration of diethylstilboestrol or its dipropionate orally or by subcutaneous implantation of solid tablets [Lewis & Turner, 1942].

The first encouraging application of this oestrogen treatment to the bovine was made by Walker & Stanley [1941], who obtained 14 and 16 lb. of milk daily from two heifers whose udders had been developed by prolonged courses of injections of diethylstilboestrol dipropionate, either alone or in conjunction with testosterone propionate. These results have since been confirmed by Reece [1943], who obtained peak daily yields of roughly 3 gal. from each of two heifers. In all these cases of heifer lactation, milking was started after the oestrogen treatment had ceased, and Reece utilized the additional stimulus of a nursing calf in order to promote a better flow of milk in the early stages of lactation.

The experiments reported below were designed to investigate the application of the tablet implantation technique of Deanesly & Parkes [1937] to the problems of oestrogen-induced lactation in maiden heifers and barren cows, and were mostly carried out under the general aegis of the Agricultural Research Council's Conference on Lactation.

## MATERIALS AND METHODS

*Animals and treatments*

Nineteen heifers, nine cows and two freemartins were used in the main experiment, each of which had proved barren after numerous services with a fertile bull. They were obtained through the co-operation of farmers in the Reading district who continued to manage them throughout the experiment. Age, breed and management thus varied considerably, but it was thought that this would be an advantage in assessing the practical value of the treatment. Each animal was fed and treated in the same way as the other animals in the same herd.

Thirteen of the heifers were  $2\frac{1}{2}$ – $4\frac{1}{2}$  years old, but only two were below 3 years. The heifers whose ages were not known had, in most cases, been unsuccessfully served several times, and it is likely that they were at least 3 years old at the start of the experiment.

This experiment suffered severely because of premature slaughter of a considerable proportion of the animals due to an outbreak of foot-and-mouth disease or on account of pelvic fracture (see below).

Each heifer received one of six treatments which had been selected to compare the efficacies of diethylstilboestrol\* (4:4'-dihydroxy- $\alpha$ : $\beta$ -diethylstilbene) and *meco*-hexoestrol (4:4'-dihydroxy- $\alpha$ : $\beta$ -diethyl-diphenylethane) in promoting lactation, when administered in the form of subcutaneous implants of relatively large numbers of small tablets. Two different sizes of tablet (25 and 15 mg.) of each substance were used, the larger tablets being administered at two dosage levels. Details of the six treatments are given in Table 1. It was planned to remove the tablets after 60 or 100 days, but in a number of cases (see Table 1) circumstances beyond our control prevented this. The nine cows and one of the freemartins were given one of four treatments involving the use of 50 mg. tablets of hexoestrol or stilboestrol, while the other freemartin received one of the six treatments used for the heifers (for details see Table 2). Milking was begun on the tenth day after implantation and continued once daily until a yield of 5 lb. was given, after which normal twice-daily milking was started.

In addition to the above animals, five heifers and five cows were implanted with large hexoestrol tablets. Five of these animals (VL 5, VL 8, VL 14, VL 15 and VL 16) were used in an investigation involving combined treatment with oestrogen and anterior-pituitary extract carried out by one of us (S. J. F.) in collaboration with Prof. F. G. Young which, it is hoped, will be fully described elsewhere. With the agreement of Prof. Young the data for VL 5, VL 8, VL 14 and VL 16, from the time of implantation until anterior-pituitary treatment was begun, and that for the whole of the lactation period of VL 15 (not given anterior-pituitary extract) are utilized here.

By the use of implants consisting of one to five large tablets the operations of insertion and removal, particularly the latter, are simplified and, for reasons given later, the accuracy of the absorption determinations increased. Particulars of the treatment accorded to these animals are given in Table 6.

\* For simplicity hereinafter referred to as stilboestrol.

*Tablet implantation and removal*

All implantations were subcutaneous. The skin of the neck was shaved and incised horizontally for 1 in., after local subcutaneous and intradermal infiltration with 5 ml. of Nupercaine. A pocket about 4 in. deep was then made between the skin and subcutaneous tissues by blunt dissection with round-ended scissors. In the case of implants consisting of small tablets, these were previously weighed and loaded into a narrow glass tube and deposited carefully at the base of the pocket, well away from the incision, which was then sutured. The tablets were not sterilized. In most cases, at removal, the bulk of the tablets was found to be encapsulated, but difficulty was often encountered in recovering all the implant, since in many cases single tablets had migrated some distance from the majority. Failure of the implants to become encapsulated, followed by tablet migration, is the most probable explanation of the inability to recover tablets, which complicated the treatment in four cases (HL 1, HL 2, HL 3 and HL 13). That such wandering could take place was clearly demonstrated in the case of heifer HL 9, in which the tablets could not be found after 100 days. They were subsequently recovered at autopsy in small groups at least 6 in. from the site of the original implant. It is believed that this tendency to migrate, together with further difficulties to be mentioned below in determining the rate of absorption, constitutes a serious objection to the use of implants consisting of large groups of small tablets inserted into the same pocket.

In four heifers (HL 8, HL 9, HL 11 and HL 15), implanted in the early stages of the work, some of the tablets were extruded and lost shortly after implantation. In these cases, as soon as the mishap was noticed, the sites were opened up and all the remaining tablets removed. Fresh implantations were then made into new pockets. In two other cases (HL 1 and HL 2) it was possible to count the extruded tablets; the original incisions were resutured and fresh tablets, equal in number to those extruded, implanted into new sites. In all these cases suitable measures were taken with the least possible delay, and it seems justifiable to assume that continuity of treatment was unaffected. Palpation of the implantation sites in three further heifers (HL 4, HL 5 and HL 7), 5-6 weeks after implantation, failed to detect any tablets. On exploration of the incised sites, few or no tablets were found. It was assumed at the time that these tablets, owing to their small size, had extruded between the sutures and a fresh implant was immediately inserted into each animal. In the light of the direct evidence of tablet migration which, as we have seen, was obtained later, it seems likely that the original implants had not been extruded but that the tablets had gradually dispersed over a wide area. It is probable therefore that over a considerable period dating from the time of re-implantation these three animals carried considerably more tablets than are given in Table 1. Where extrusion of tablets occurred it was probably because the subcutaneous pocket was not made sufficiently deep. When deeper pockets were made the trouble was no longer experienced.

No difficulties due to tablet extrusion or location of the implant at removal were encountered when large tablets were used. For these the technique was similar, though involving less blunt dissection.

*Absorption data*

The recovered tablets were thoroughly washed in water and any adhering tissue removed. After drying over calcium chloride they were equilibrated in air and weighed. In no case in which small tablets were used was the theoretical number of whole, unfragmented tablets recovered. Consequently the undamaged tablets were always weighed separately and the probable weight of the whole implant calculated on the assumption that all of the component tablets had undergone equal absorption. This figure was used for calculation of the total amount of oestrogen absorbed. Large tablets were recovered intact in all cases.

The occurrence of ghost formation [Folley, 1942] was demonstrated in one or two recovered batches of small tablets and in some of the large tablets, but no determinations of true absorption values, allowing for the presence of ghosts in the tablets, were made.

*Udder preparations*

The udders were removed from a number of the treated animals at slaughter and fixed in 10% formalin. Longitudinal slices were cut about  $\frac{1}{2}$  in. thick, passing through the fore and hind teats on either side. These whole slices were then stained with borax carmine, differentiated in 70% alcohol containing 5% hydrochloric acid and finally preserved in 4% formalin.

## RESULTS

*Implantation with 15, 25 or 50 mg. oestrogen tablets**Lactation*

The lactation curves are presented in Figs. 1-6 and the maximum and total yields in Tables 1 and 2. Although the number of animals given any one treatment was too small to obtain results of statistical significance, certain conclusions can be drawn which may tentatively be assumed to have a validity for other such heterogeneous groups of barren cattle.

Of first importance is the variability in response both throughout the experiment as a whole and also in relation to any one treatment, a variability embracing both the degree and rate of udder development and the degree, duration and rate of onset of lactation. In particular, maximum daily yields varied from  $\frac{1}{2}$  to 30 $\frac{1}{4}$  lb. and full lactation yields from 5 to 740 gal. (CL 9 and CL 6). Further, it may be adduced that no difference exists between the capacities of stilboestrol and hexoestrol for initiating lactation under the conditions of these experiments and also that the smaller implants used, amounting to approximately 2.5 g., were as satisfactory as the larger ones (5.0 g.) for this purpose. This is of great importance in view of the nymphomaniac symptoms, to be referred to later, which make it seem desirable to reduce the daily oestrogen intake to a minimum (see following section). Lastly—and a point which also bears on the advisability of minimum oestrogen absorption—it seems probable that no advantage accrues from leaving the oestrogen tablets *in situ* once the lactation curve has reached its highest level; on the contrary, removal of the tablets at this point frequently caused a sudden rise in milk yield indicating a transition of the oestrogenic action from a stimulative to an inhibitory phase, a transition intervening at different production levels for different animals (cf. HL 5, HL 6

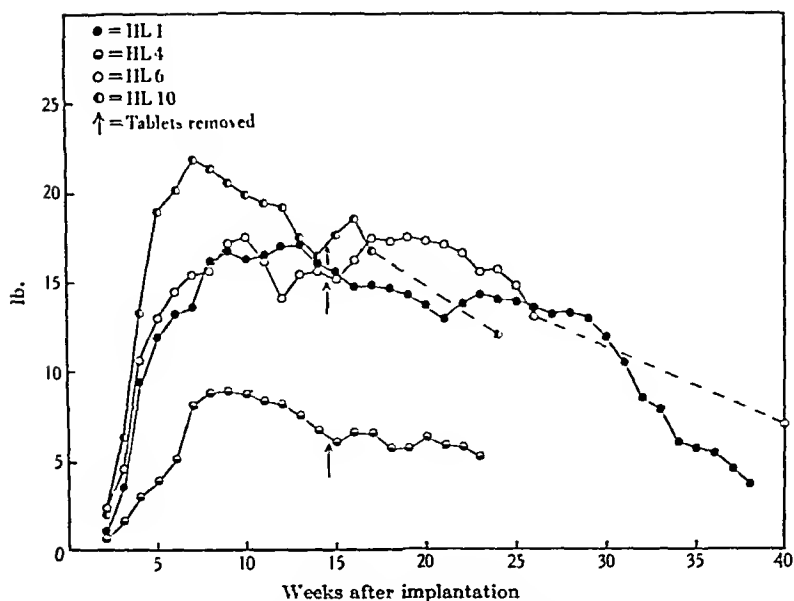


FIG. 1. Lactation curves for heifers implanted with  $200 \times 25$  mg. tablets of hexoestrol. (Mean daily yields for 7-day periods.)

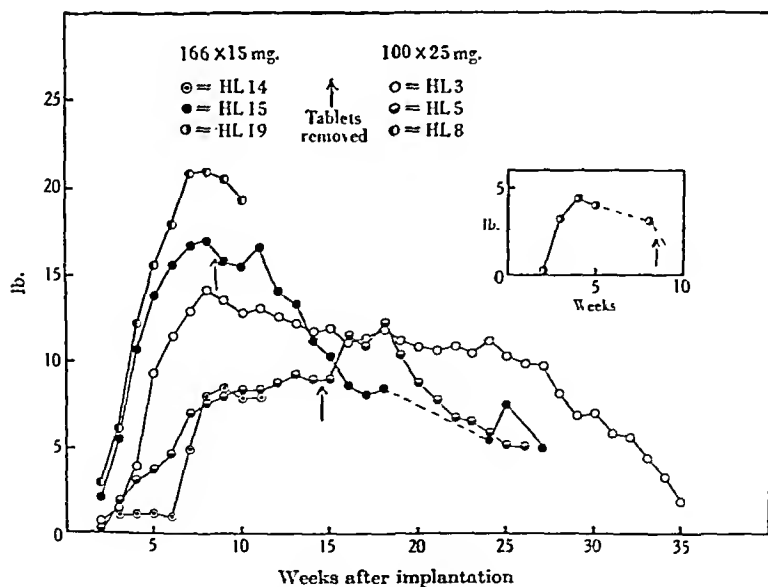


FIG. 2. Lactation curves for heifers implanted with  $166 \times 15$  mg. or  $100 \times 25$  mg. tablets of hexoestrol. (Mean daily yields for 7-day periods.)



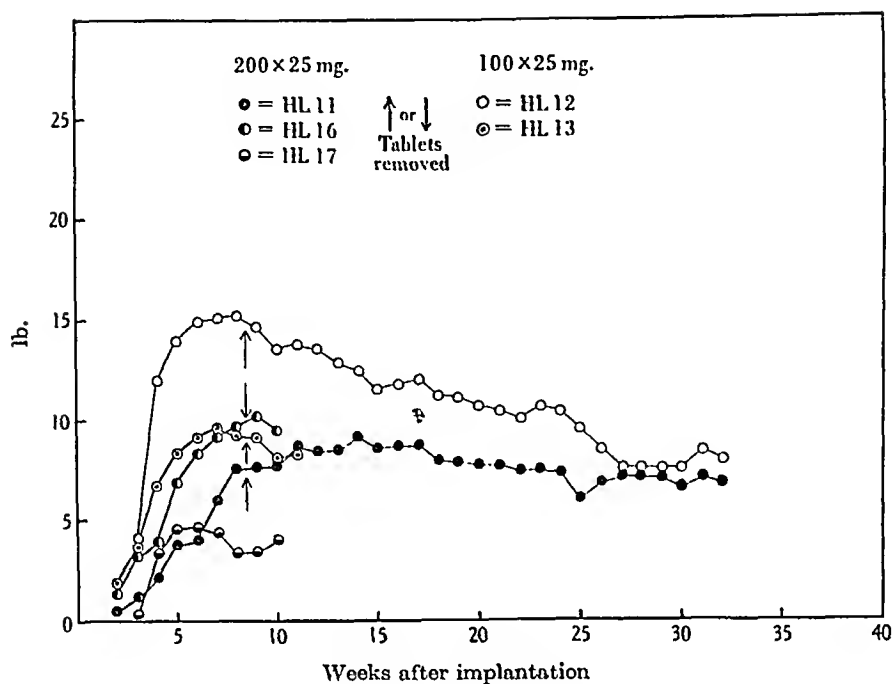


FIG. 3. Lactation curves for heifers implanted with  $200 \times 25$  mg. or  $100 \times 25$  mg. tablets of diethylstilboestrol. (Mean daily yields for 7-day periods.)

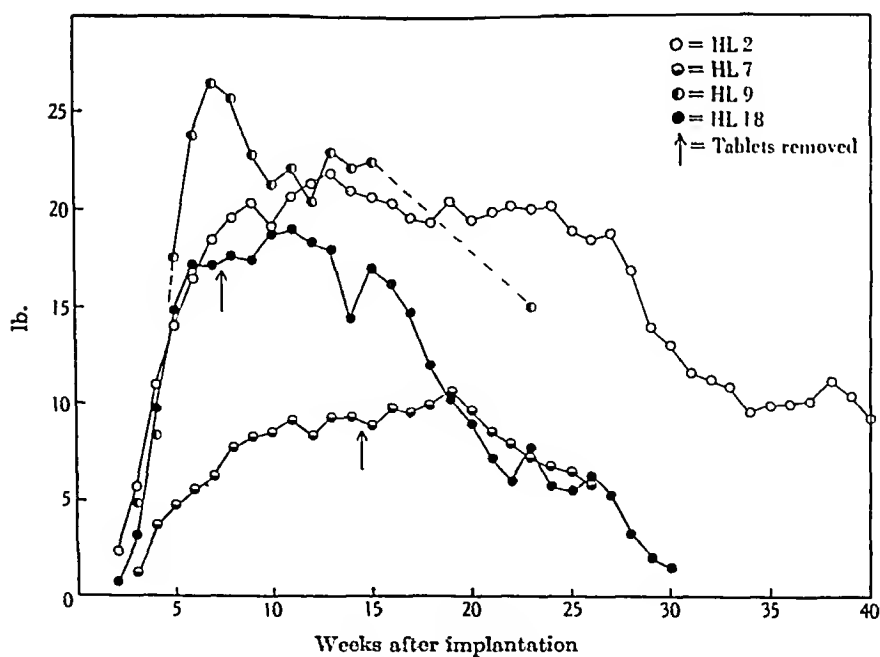


FIG. 4. Lactation curves for heifers implanted with  $166 \times 15$  mg. tablets of diethylstilboestrol. (Mean daily yields for 7-day periods.)

# INDUCED LACTATION

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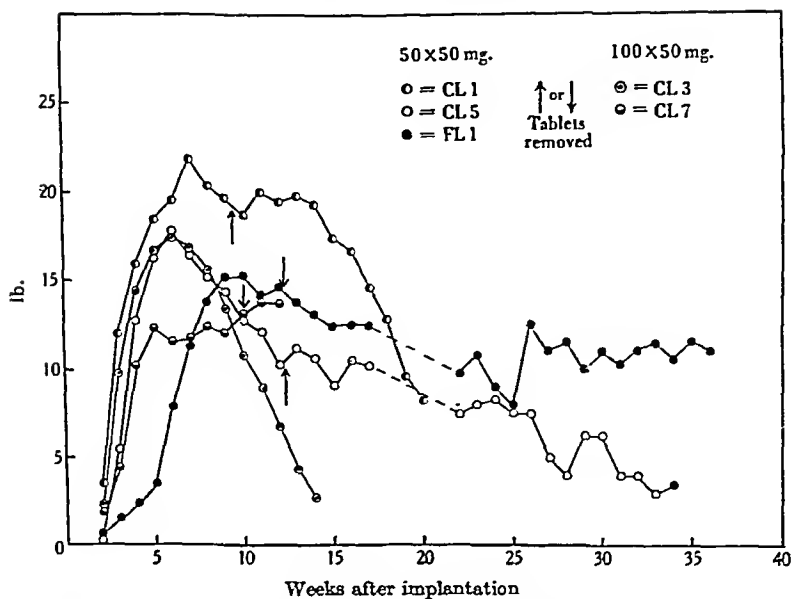


FIG. 5. Lactation curves for cows, and one freemartin, implanted with 50x50 mg. or 100x50 mg. tablets of hexoestrol. (Mean daily yields for 7-day periods.)

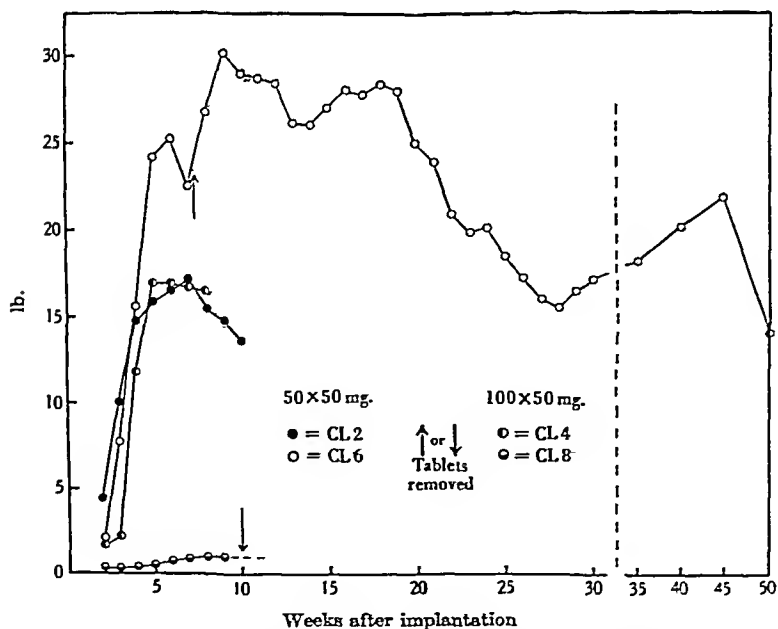


FIG. 6. Lactation curves for cows implanted with 50x50 mg. or 100x50 mg. tablets of diethylstilboestrol. (Mean daily yields for 7-day periods.)

and CL 6). Of special interest in this respect is the lactation curve of cow CL 3, from which the tablets could not be removed owing to an outbreak of foot-and-mouth disease on a neighbouring farm. The lactation curve of this animal reached a peak of just over 17 lb. daily and thereafter fell rapidly (Fig. 5). It is instructive to compare this curve with that of cow CL 5 (Fig. 5), which showed the same phenomenon until the tablets were removed at 86 days. After removal of the tablets there was a clear arrest in the rate of decline of lactation. The limited data available suggest that dry cows and maiden heifers respond in the same way to the treatment and it is of interest to note the considerable yield of one of the freemartins (FL 1). The second freemartin failed to secrete and showed no udder development after 31 days of treatment.

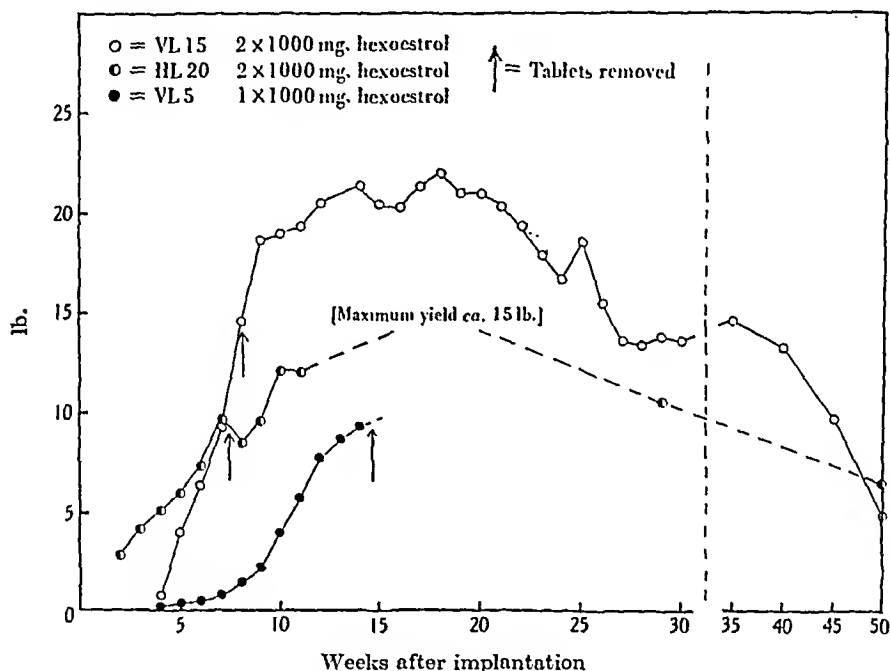


FIG. 7. Lactation curves for heifers implanted with 2 × 1000 mg. or 1 × 1000 mg. tablets of hexoestrol. (Mean daily yields for 7-day periods.)

A longitudinal section of the right half of the udder of HL 6, a heifer which was slaughtered because of pelvic fracture after having produced approximately 3500 lb. of milk in 273 days (see Table 1), is shown in Plate 1, fig. 8 (facing p. 22). The teats and gland cisterns were well developed and there was an extensive alveolar system. This specimen is typical of others obtained from those heifers which were slaughtered.

#### Absorption data

The mean daily absorption ranged from 8.0 to 23.9 mg. (Tables 3, 4). Since no allowance was made for the weights of the ghosts undoubtedly present in these tablets, the values given must be less than the true values, possibly by at least 20% [Folley, 1942, 1944]. The accuracy of these figures is further decreased by the fact that in all cases fragmentation of some tablets occurred *in situ*, probably because

# INDUCED LACTATION

Table 1. *Details of treatment and milk yields of heifers implanted with small tablets of synthetic oestrogens*

No.	Breed	Implant	Dura- tion of treat- ment days	Maxi- mum daily yield lb.*	Total recorded yield lb.	Remarks
11L 1	Shorthorn	200 x 25 mg. hexoestrol	?	17	3104	Partial re-implant at 16 days. No tablets found after 100 days
11L 4	Crossbred	"	100	9	945	Re-implanted after 30 days
11L 6	Shorthorn	"	104	17½	o. 3500	Pelvic fracture sustained; slaughtered at end of lactation
11L 10	Friesian	"	103	22	c. 2000	—
11L 3	Shorthorn	100 x 25 mg. hexoestrol	?	14	2108	No tablets found after 100 days
11L 5	"	"	104	12½	1208	Re-implanted after 42 days
11L 8	"	"	78	21	1079	Re-implanted after 28 days. Pelvic fracture sustained; slaughtered during lactation
11L 14	"	160 x 15 mg. hexoestrol	80	9½	298	Slaughtered during lactation because of foot-and-mouth disease
11L 15	"	"	60	17	1792	Re-implanted after 15 days
11L 19	"	"	60	4½	150	—
11L 11	"	200 x 25 mg. diethylstilboestrol	00	9	1400	Re-implanted after 20 days
11L 16	"	"	00	10½	400	Slaughtered during lactation because of foot-and-mouth disease
11L 17	Guernsey	"	73	4½	197	Pelvic fracture sustained; slaughtered during lactation?
11L 12	Shorthorn	100 x 25 mg. diethylstilboestrol	60	15	2298	Only 8 tablets found after 01 days; slaughtered during lactation because of foot-and-mouth disease
11L 13	"	"	?	9½	510	—
11L 2	"	160 x 15 mg. diethylstilboestrol	?	22	4310	Partial re-implant at 13 days. No tablets found after 100 days
11L 7	"	"	104	19½	1294	Re-implanted after 42 days
11L 9	Friesian	"	106	20½	o. 2800	Re-implanted after 35 days. Pelvic fracture sustained; slaughtered at end of lactation
11L 18	Shorthorn	"	53	19	2215	—

\* Mean for 7-day period.

Table 2. *Details of treatment and milk yields of cows and freemartins implanted with small tablets of synthetic oestrogens*

No.	Breed	Implant	Dura- tion of treat- ment days	Dura- tion of recorded lactation days	Maxi- mum daily yield lb.*	Total recorded yield lb.	Remarks
CL 3	Guernsey	100 × 50 mg. hexoestrol	114	93	17½	980	—
CL 7	"	"	69	83	14	884	Pelvic fracture sustained; slaughtered during lactation
CL 1	Shorthorn	50 × 50 mg. hexoestrol	66	133	21½	2153	—
CL 5	Friesian	"	86	231	17½	2076	—
CL 9	Shorthorn	"	61	79	½	< 50	—
CL 4	"	100 × 50 mg. diethylstilboestrol	63	52	17	617	Pelvic fracture sustained; slaughtered during lactation
CL 8	"	"	69	101	1	< 100	—
CL 2	"	50 × 50 mg. diethylstilboestrol	70	60	17	819	Slaughtered during lactation because of foot-and-mouth disease
CL 6	"	"	51	365	30½	7395	—
FL 1	Ayrshire	50 × 50 mg. hexoestrol	86	246	15½	2573	Freemartin
FL 2	"	100 × 25 mg. diethylstilboestrol	31	0	0	0	Freemartin

\* Mean for 7-day period.

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Table 3. *Absorption data for heifers implanted with small tablets of synthetic oestrogens*

No.	Implant*	Wt. of implant mg.	Dura- tion of treat- ment days	Wt. of recovered implant mg.	No. of whole tablets recovered	Wt. of whole tablets recovered mg.	Final wt. implant (encl.) mg.	Estimated total absorption mg.	Estimated mean daily absorption mg.	Total recovery expressed as no. of whole tablets†	Estimated total recovery %‡
HL 1	200 × 25 mg. Hex.	5015	—	—	—	—	—	—	—	—	—
HL 4	"	4001	04	3457	163	2878	3701	1200	18.8	184	92.0
HL 6	"	4000	104	3045	07	1030	3300	1030	15.8	181	90.5
HL 10	"	5008	103	3187	103	2700	3313	1005	10.5	102	90.0
HL 3	100 × 25 mg. Hex.	2537	—	—	—	—	—	—	—	—	—
HL 5	"	2527	02	1000	01	1040	1715	812	13.1	07	97.0
HL 8	"	—	—	—	—	—	—	—	—	—	—
HL 14	100 × 15 mg. Hex.	2348	—	—	—	—	—	—	—	—	—
HL 15	"	2330	45	1206	137	1206	1570	760	17.1	137	82.5
HL 10	"	2240	06	1533	103	1533	1502	078	11.3	103	88.2
HL 11	200 × 25 mg. Daos.	4888	31	4160	106	4160	4245	043	20.7	106	98.0
HL 10	"	4034	00	3413	175	3130	3577	1357	22.0	101	95.5
HL 17	"	4014	73	2004	160	2520	3360	1554	21.3	173	80.5
HL 12	100 × 25 mg. Daos.	2408	00	1815	03	1815	1052	510	8.0	03	93.0
HL 13	"	—	—	—	—	—	—	—	—	—	—
HL 2	100 × 15 mg. Daos.	—	—	—	—	—	—	—	—	—	—
HL 7	"	2371	02	1217	153	1135	1231	1140	18.4	164	98.8
HL 0	"	2370	101	560	131	500	634	1736	10.8	148	80.2
HL 18	"	2370	53	1508	137	1508	1036	434	8.2	137	82.5

\* Hex. = hexoestrol; Daos. = diethylstilboestrol.

†  $\frac{\text{Wt. of recovered implant} \times \text{No. of whole tablets}}{\text{Wt. of whole tablets}}$       ‡  $100n_1/n_2$ , where  $n_1$  = no. of tablets implanted.

Table 4. *Absorption data for cows and freemartins implanted with small tablets of synthetic oestrogens*

No.	Implant*	Wt. of implant mg.	Dura- tion of treat- ment days	Wt. of recovered implant mg.	No. of whole tablets recovered	Wt. of whole tablets recovered mg.	Final wt. of whole implant (c.l.c.) mg.	Estimated total absorption mg.	Estimated mean daily absorption mg.	Total recovery expressed as no. of whole tablets†	Estimated total recovery‡ %
CL 3	100 × 50 mg. Hex.	4711	114	2814	29	920	3173	1538	13.5	89	89.0
CL 7	"	4689	69	3357	63	2190	3476	1213	17.6	97	97.0
CL 1	50 × 50 mg. Hex.	2365	66	1680	34	1196	1759	606	9.2	48	96.0
CL 5	"	2380	86	1254	0	—	—	—	—	—	—
CL 9	"	2333	61	1841	35	1290	1843	490	8.0	50	100.0
CL 4	100 × 50 mg. Daes.	5001	63	3410	85	3024	3558	1503	23.9	96	96.0
CL 8	"	5047	68	3798	71	2776	3909	1138	16.5	97	97.0
CL 2	50 × 50 mg. Daes.	—	—	—	—	—	—	—	—	—	—
CL 6	"	2515	51	1346	49	1346	1374	1141	22.4	49	98.0
FL 1	50 × 50 mg. Hex.	2374	86	922	30	764	1274	1100	12.8	36	72.0
FL 2	100 × 25 mg. Daes.	2457	31	1870	98	1870	1908	549	17.7	98	98.0

\* Hex. = hexoestrol; Daes. = diethylstilboestrol.

† 100n<sub>2</sub>/n<sub>1</sub>, where n<sub>1</sub> = no. of tablets implanted.‡  $\frac{\text{Wt. of recovered implant} \times \text{No. of whole tablets}}{\text{Wt. of whole tablets}}$ .

of their mutual chafing action, necessitating the calculation of the final weight of the implant from the weight of the intact tablets, assuming that all had undergone equal absorption. This assumption, however, is at the best only an approximation to the truth. Members of batches of small tablets are often unequally absorbed, as can be seen from Plate 1, fig. 9 (facing p. 22), which shows the largest and smallest tablets recovered from an implant originally consisting of tablets of equal size. The uncertainty in the absorption determinations arising from tablet fragmentation is, however, probably not excessive, since at least 75% of the original tablets were recovered intact in most cases.

As can be seen from the last two columns of Tables 3 and 4, the total material (intact tablets and debris) recovered from any given implant, expressed in terms of the number of tablets of weight equal to the mean weight of those tablets recovered intact, usually amounted to over 90% of the theoretical value. This indicates that in most cases little or no material was missed.

The mean daily absorption rates for stilboestrol and hexoestrol implants at the two dosage levels are given in Table 5. In spite of the inaccuracies in the absorption data

Table 5. *Rates of absorption in the bovine of implants of small tablets of synthetic oestrogens*

Substance	Approximate total initial wt. of implant g.	No. of animals	Mean daily absorption; average for all animals mg.
Diethylstilboestrol	2.5	6	14.4
"	5.0	5	21.0
Hexoestrol	2.5	6	11.9
"	5.0	5	16.4

discussed above and the small size of the groups, it seems justifiable to conclude that the daily absorption from the 2.5 g. implants was considerably smaller than from the 5.0 g. implants, and that in the bovine, stilboestrol is absorbed appreciably faster than hexoestrol, contrary to the finding of Forbes [1943] that the times required for 90% absorption of tablets of these two substances were not very different in the rat.

#### *Physiological and anatomical disturbances associated with oestrogen implantation*

Nymphomaniac behaviour was shown by all animals implanted with oestrogens except one or two which were stall-fed or pastured by themselves. The symptoms were usually noticed within 14 days of treatment and although far more persistent and severe, were limited in their nature to the characteristic 'mounting' behaviour of the cow at oestrus. Associated with this syndrome were two further features. The first, relaxation of the sacro-sciatic and sacro-iliac ligaments with attendant raising of the tail head and lowering of the coxal tubers, was of general occurrence; while the second, fracture of the pelvis, occurred in six animals (four heifers and two cows, Tables 1, 2), or 20% of the number treated. The occurrence of these fractures, the serious practical implications of which cannot be underestimated, is more fully discussed in an addendum to this paper.

Regular examination of the ovaries by rectal palpation showed that the effect of the oestrogen treatment was to render the ovaries inactive (see Plate 1, fig. 10, facing p. 22).



Table 6. *Details of treatment and milk yields of heifers and cows implanted with large hexoestrol tablets*

No.	Cow or heifer	Breed	Implant	Dura- tion of treat- ment days	Dura- tion of lactation days	Maxi- mum daily yield lb.*	Total yield lb.	Remarks
VL 6	Heifer	Jersey	5 x 1000 mg. hexoestrol	63	—	< 1	—	—
VL 8	Cow	"	"	63	—	< $\frac{1}{2}$	—	Subsequently treated with ox-pituitary extract
CL 10	"	Shorthorn	2 x 1000 mg. hexoestrol	101	340	c. 25	7	Suckled calves. Still lactating
CL 11	"	"	"	101	—	0	0	—
VL 16	"	Crossbred	"	56	—	0	0	—
VL 15	Heifer	"	"	55	329	22	4888	Subsequently treated with ox-pituitary extract
VL 14	Cow	"	"	40	—	< $\frac{1}{2}$	—	—
HL 20	Heifer	Guernsey	"	51	365	12	c. 3500	Subsequently treated with ox-pituitary extract
HL 21	"	"	"	51	28	1 $\frac{1}{2}$	20	—
VL 5	"	Red Poll	1 x 1000 mg. hexoestrol	102	83	10 $\frac{1}{2}$	315	Pelvic fracture probable but unconfirmed
								Subsequently treated with ox-pituitary extract

\* Mean for 7-day period.

Table 7. *Absorption data for animals implanted with large hexoestrol tablets*

No.	Implant	Wt. of implant mg.	Duration of treatment days	Wt. of recovered implant mg.	Total absorption mg.	Mean daily absorption mg.
VL 6	5 x 1000 mg. hexoestrol	5240	63	4280	960	15.2
VL 8	"	5240	63	4380	800	13.7
CL 10	2 x 1000 mg. hexoestrol	1906	101	1529	377	3.7
CL 11	"	2061	101	1535	526	5.2
VL 16	"	1910	56	1620	290	5.2
VL 15	"	1960	55	1650	310	5.6
VL 14	"	1920	55	1675	245	4.5
HL 20	"	2015	51	1644	371	7.3
HL 21	"	1998	51	1698	300	5.9
VL 5	1 x 1000 mg. hexoestrol	1121	102	849	272	2.7

Average = 14.5

Average = 5.3

There was no evidence of luteinization such as occurs in the rat under prolonged dosage with oestrogen [Hohlweg, 1934]. The relatively high daily absorption of oestrogen in the present experiments would appear to have inhibited all pituitary gonadotrophic function.

Frequently, but not invariably, removal of the implant was followed by a burst of pituitary follicle-stimulating activity. In a few cases this was sufficient to cause the production of cystic follicles which usually disappeared eventually. More characteristically the ovaries smoothly renewed their periodicity within about two months of the cessation of treatment. The marked evidence of follicle stimulation frequently observed suggests that the circulating oestrogen inhibited the release of gonadotrophin rather than its formation.

Owing to the premature slaughter of ten of the animals in the main experiment because of foot-and-mouth disease or pelvic fracture, the effect of treatment on subsequent fertility could only be investigated in nine heifers and two cows. Of these, one heifer has become pregnant.

#### *Implantation with 1000 mg. oestrogen tablets*

The milk yields given in Table 6 clearly show that variability of response was also a main feature of the treatment involving implantation with small numbers of large hexoestrol tablets. There were four cases in which appreciable responses were obtained. In one of these (CL 10), no figures are available on which to base an estimate of the maximum and total yields, since the animal was used for suckling a succession of calves. This in itself indicates that the yield must have been considerable. On one occasion on which the daily yield was measured she gave 25 lb.

One pelvic fracture was encountered in this series and nymphomania was common, though less noticeable than in the main group.

The absorption data (Table 7) are more reliable for these animals and suggest that the small tablet implants resulted in an unnecessarily high daily absorption for the effective stimulus to udder growth and lactation.

#### DISCUSSION

The present experiments indicate that in a useful proportion of cases, copious lactation may be initiated in maiden heifers and dry cows by the subcutaneous implantation of solid tablets of synthetic oestrogens. Most of the successful responses were obtained when large batches of small tablets were implanted. Experiments involving the implantation of large hexoestrol tablets were too few to estimate the efficacy of such convenient implants for inducing lactation, though considerable responses were obtained in four cases. There seems to be no reason why the implantation of suitable numbers of large tablets should not prove as satisfactory as the use of batches of small ones, and it can be definitely stated that large tablets can be far more easily and quantitatively removed, partly because they are not subject to the fragmentation *in situ* which complicates the technique and, moreover, renders the determination and interpretation of absorption values so difficult with implants of small tablets.

The lactation and tablet-absorption data from the main experiment, limited though they are, indicate that there is no advantage in using implants giving a daily

absorption of more than about 12 mg. of hexoestrol or, assuming both substances are equally active, stilboestrol. From the mean absorption values for large tablets given in Table 7 it would appear that this daily absorption rate would be attained by the use of implants consisting of  $4 \times 1000$  mg. tablets of hexoestrol or, since stilboestrol is absorbed faster, perhaps  $3 \times 1000$  mg. tablets of stilboestrol. It is well to point out, however, that it is doubtful how far conclusions adduced from experiments with small tablets are applicable to experiments involving large tablets, since the absorption of the latter is a more complex phenomenon than the absorption of single small tablets [Folley, 1943, 1944]. Actually, even the above amount may be excessive, since considerable responses were obtained in three cases by the use of two large tablets giving a mean daily absorption of the order of 5 mg. of hexoestrol daily. This figure is, of course, subject to error due to neglect of the ghost correction [Folley, 1942] and because the absorption rate of large hexoestrol tablets is not uniform over the whole absorption period [Folley, 1943, 1944].

An outstanding feature of the results throughout was the variability in response, the reasons for which can only be conjectured. The explanation may partly lie in uncontrollable variations in the rates of absorption of the tablets, which, these experiments have shown, must be expected when implants consisting of batches of small tablets subject to fragmentation *in situ* are used. Absorption is likely to proceed faster from tablet debris than from undamaged tablets. Furthermore, considerable variations in response must, of course, be expected among a heterogeneous population of experimental animals which are far from uniform in respect of such factors as age, nutritional status and genetic capability for lactation. Whatever may be the true explanation, our results suggest that no standard treatment is likely to elicit the best possible responses from more than a proportion of a given group of bovines.

Two main processes are involved in the lactation response, growth of the mammary gland, followed by initiation and maintenance of lactation. Leaving aside the controversial question whether mammary growth follows from the direct action of ovarian hormones or whether the anterior pituitary is involved, it seems possible, since no evidence exists to the contrary, that, by analogy with numerous experiments on small animals [for review, see Folley, 1940] progesterone is needed as well as oestrogen for full alveolar development in the bovine. As has been pointed out previously [Folley, 1940] the interpretation of evidence regarding the necessity or otherwise for the participation of progesterone in mammary growth has been complicated by the isolation of progesterone from the adrenal cortex [Beall & Reichstein, 1938]. Since no corpora lutea were found during treatment in the ovaries of the bovines used in the present experiments, the only source of progesterone would therefore appear to be the adrenal cortex. On the assumption that progesterone is necessary for full mammary development in the bovine, it is conceivable that insufficient mammary alveolar development due to a deficiency of progesterone may have been a limiting factor in some cases where the lactation response was poor. Alternatively, even in the presence of ample progesterone, if the oestrogen dosage was excessive or too prolonged, mammary development may have been curtailed or of an abnormal type so that the secretory power of the gland would have been suboptimal. Inhibition of mammary growth in various species by high doses of oestrogens has been described by Gardner [1941], while the dependence of optimal mammary alveolar development

in the rabbit upon a rather delicate balance between oestrogens and progesterone has been demonstrated by Lyons & McGinty [1941] and Scharf & Lyons [1941].

With regard to the initiation and maintenance of lactation, the present work may be taken as providing confirmatory evidence in favour of the view [Folley, 1941; Folley *et al.* 1941] that under suitable circumstances oestrogen will stimulate the production of lactogenic and galactopoietic hormones by the anterior pituitary provided the dosage is not too high. The recent work of Meites & Turner [1942], who showed that oestrogen treatment increases the prolactin content of the pituitary, might also be taken as evidence in support of this theory, provided the observed increases signify increased secretion of prolactin and not merely inhibition of its release. It is possible that some cases of partial or complete failure of lactational response in these experiments might be explained on the basis of a lack of pituitary lactogenic or galactopoietic hormones due, perhaps, to a general functional inhibition of the anterior pituitary by the excessive or too-prolonged action of oestrogen. Evidence in support of this hypothesis is provided by the experiments of Folley & Young [1941, and further unpublished work], who found that injections of anterior-pituitary extracts would further increase the peak yields of goats and bovines in which lactation had been induced by treatment with synthetic oestrogens. Particularly significant for the point under discussion were one or two cases in which animals which had failed, under the stimulation of oestrogen alone, to produce more than negligible amounts of secretion, came into copious lactation in response to anterior-lobe extract.

The above considerations provide a reasonable explanation of the results in cases in which removal of the implant was followed by a rise in milk yield or in which a rapid decline in the lactation curve was associated with failure to remove the tablet (e.g. CL 3 and CL 5). In these cases the degree or duration of the oestrogen stimulus must have been of sufficient magnitude eventually to inhibit the galactopoietic functions of the pituitary. These facts serve to emphasize the dependence of an optimal lactational response on a very close control of oestrogen dosage and duration of treatment.

The nymphomaniac syndrome, which to a varying degree was exhibited by most animals under experiment, and which in a disturbingly high proportion of cases culminated in fracture of the pelvis necessitating the slaughter of the affected animals, constitutes a serious and undesirable side-effect of prolonged oestrogen treatment. The cause of the pelvic fractures will not be discussed in detail here since it is dealt with in an addendum to this paper. The fractures appeared to have occurred during coital mimicry, a usual feature of the nymphomaniac syndrome, and since there was no evidence of decalcification of the pelvis it is probable that they would not have occurred had the animals in question been kept under isolation during the period of treatment.

#### SUMMARY

1. Lactation was induced in nulliparous heifers and dry cows by the subcutaneous implantation of large numbers of small tablets of diethylstilboestrol or hexoestrol. Individual variations in response were large, but in some cases the lactation curve was comparable with that of normal lactation. The best response was obtained with a dry cow which gave a maximum yield of 30½ lb. daily and a total yield of 7400 lb

2. Implants totalling 5.0 and 2.5 g. were equally effective, and there appeared to be no difference in efficacy between diethylstilboestrol and hexoestrol.

3. Copious lactation was also induced in nulliparous heifers and dry cows by the subcutaneous implantation of one or two tablets of hexoestrol weighing approximately 1000 mg.

4. Tablets of diethylstilboestrol implanted into bovines were absorbed faster than tablets of hexoestrol.

5. The principal drawbacks associated with the use of implants consisting of large batches of small tablets were tablet fragmentation and migration. Neither happened when large hexoestrol tablets were used.

6. The oestrogen treatment was, in most cases, accompanied by a nymphomaniac syndrome, which, in 20 % of the experiments in which small tablets were implanted, culminated in pelvic fracture.

For the provision and care of the experimental animals we are indebted to numerous farmers in the Reading district and elsewhere. Our best thanks are also due to Mr A. T. Cowie for assistance with the implantation of tablets and with the ovarian palpations; to Prof. F. G. Young for agreeing to our using the data for five of the animals implanted with large tablets; to Mr R. E. Glover for implanting the tablets into two of these latter animals; and to Mr R. W. Hill of the Ministry of Food for providing facilities for the recovery of udders at slaughter. The work was carried out during the tenure by one of us (F. H. M.) of a research grant from the Agricultural Research Council who also provided the small oestrogen tablets.

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## ADDENDUM

FRACTURE OF THE PELVIC BONES IN BOVINES IMPLANTED  
WITH TABLETS OF SYNTHETIC OESTROGENS

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(Received 17 December 1943)

*Description of fractures*

Cow CL 4 was slaughtered as she was unable to stand. Fracture of the pelvis was suspected. Post-mortem examination revealed that the right ilium was fractured transversely at approximately the junction of the shaft and the wing. The fractured ends were becoming rounded but there was no callus formation. The left ilium was fractured in the same region but more obliquely and there was slight callus formation. The ventral sacro-iliac ligaments on both sides were abnormally relaxed and the sacro-iliac articulations were congested.

Heifer HL 17 showed very slight symptoms; the fracture was diagnosed from the displacement of the right coxal tuber. The animal was slaughtered at the owner's request in case the symptoms became acute. A post-mortem examination was made and the fracture was found to extend obliquely from the sacral tuber across the wing of the ilium to the lateral border in the region of the junction of the wing and shaft. The periosteum was intact on the pelvic surface but was ruptured on the gluteal surface. The various pelvic ligaments were relaxed and the sacro-iliac articulations abnormally mobile.

Heifer HL 9 was not slaughtered at the time the fracture was first diagnosed, but 5 months later the symptoms became acute and slaughter was necessary. Autopsy revealed that both ilia had been fractured but that the bones had united. The right ilium had fractured obliquely from the cranial border of the shaft to the caudal end of the ischiatic spine. There had been backward displacement of the ilium and the bone had united with large callus formation which overhung the acetabulum but did not involve the joint. The left ilium had fractured transversely through the acetabular end of the shaft. In the fresh state the callus appeared firm, but when boiled the bone came apart as calcification was not quite complete on this side. The right sacro-iliac ligaments were found to be recently ruptured—probably the reason for the lameness becoming suddenly acute.

Heifer HL 6 developed symptoms of fracture soon after implantation but she was not slaughtered till the end of lactation. The right ilium had been fractured from the acetabular end of the shaft obliquely across to the lesser sciatic notch. There had been backward displacement of the ilium, and large callus formation united the bones and overhung the acetabulum.

Heifer HL 8 fractured her hip bone (diagnosis by farmer's own veterinary surgeon). She was slaughtered, but unfortunately owing to lack of notification the carcass was not examined.

Heifer HL 21 fractured the left ilium in the region of the coxal tuber. She was not slaughtered so the exact position of the fracture is not known.

Cow CL 7 showed no symptoms of fracture when alive. She was slaughtered for economic reasons and the slaughterman reported a fracture of the hip bone.

### *Discussion*

In considering the causes of these fractures it is interesting to note the great similarity in the clinical symptoms shown by oestrogen-implanted cows and heifers with those shown by cows suffering from 'nymphomania' due to cystic ovaries. Although symptoms of extreme severity which are occasionally encountered in the 'natural' disease were not observed in the experimental animals, the morphological changes and the sexual behaviour which tends to become more male than female, were in general the same in the 'induced' as in the 'natural' disease. Fractures of the pelvic bones are frequent in nymphomania [Tutt, 1933; Williams, 1939]. It has been suggested that the fractures in 'nymphomania' are predisposed by a rarefaction of the bones; there does not appear to be any reliable experimental evidence in support of this view.

In the experimental animals, while the immediate cause of fracture would undoubtedly appear to be trauma sustained during coital mimicry, two predisposing factors would seem worthy of attention. The oestrogen may either cause an alteration in structure and/or chemical composition of the bones, or it may cause the bones, by alteration of their angular relationships with each other, to be placed in positions less favourable to withstand stresses and strains.

### *Structure and composition of affected bones*

The effect of oestrogens on bone has been extensively studied in the smaller laboratory animals. The literature pertaining to this subject has recently been reviewed by Gardner & Pfeiffer [1943]. It is known that hyperealcification of the skeleton with partial or complete obliteration of the marrow cavities occurs in rats and chickens [Zondek, 1937] and in mice [Gardner & Pfeiffer, 1938] following prolonged oestrogen treatment, and simultaneously in mice there is an absorption of the pubic and ischial bones and replacement of the symphyseal cartilage with ligamentous tissue [Burrows, 1935; Gardner, 1935]. I have been unable to find any reference to experiments on large animals.

### *X-ray examination*

The pelvic bones from animals HL 17, HL 9 and CL 4 were boiled free of soft tissue and the iliac portions were X-rayed. The radiographs were submitted to Prof. G. F. Boddie (Royal (Dick) Veterinary College, Edinburgh) for examination and interpretation. Prof. Boddie reported that he was unable to find any evidence of rarefaction in the bones from the experimental animals, in fact, in the case of HL 17 and CL 4 the cortical bone appeared slightly more dense than in control

bones. In the bones where callus formation had taken place, the X-ray appearance was indicative of effective deposition of calcium. From the examination of the radiographs he was of the opinion that the fractures were not due to decalcification but were rather traumatic in origin.

### *Chemical analyses*

Owing to the size of the bones it was not practicable to carry out chemical analyses of the entire bones, so comparable portions of bone from the same region of the shafts of the ilia were taken and analysed for calcium and phosphorus and total nitrogen. Specific-gravity measurements were also made. The results (see Table 1) show no significant difference between the control and experimental group.

Table 1. *Analyses of bone from experimental and control animals*

	Total					Total			
	Ca %	P %	N %	Sp.-gr.		Ca %	P %	N %	Sp.-gr.
Control					Experimental				
1	23.1	10.8	4.75	2.09	HL 17	22.6	10.3	4.49	2.05
2	24.1	10.9	4.39	2.01	CL 4	23.1	10.5	4.31	2.12
3	23.5	10.7	4.41	1.99	HL 9	24.0	10.6	4.53	2.04
4	24.7	11.1	4.31	2.04					

### *Breaking strength*

Bell & Cuthbertson [1943] have shown that, in rats, oestrogens induced the formation of slightly heavier and stronger femora than normal, but there was no alteration in the quality of the bone as indicated by the breaking stress. It was hoped to make breaking tests on these bovine bones. Owing to the fact that the material was limited it was not possible to use the whole bones. Expert advice was obtained on the possibility of using small portions of the bones as 'test-pieces' and specifications of these were laid down, but it was not possible to fulfil these and the idea had to be abandoned.

### *Conclusions*

There is thus at present no evidence that oestrogens produce any appreciable change in the composition or structure of the pelvic bones of cows in the relatively short dosage periods required for the induction of lactation.

### *Alterations in the morphology of the hindquarters*

Oestrogen implantation produces marked anatomical changes in the pelvic region. The degree of alteration varies in different animals and in general is less marked in well-nourished animals. There is a general relaxation of the pelvic ligaments and the pelvis becomes tilted forward on the head of the femur, thus depressing the coxal and sacral tubers and elevating the ischial tubers. In addition, the sacro-iliac ligaments are relaxed and the sacrum is tilted forwards, the lumbo-sacral articulation being bent ventrally and the sacro-coccygeal articulation raised. The sacro-sciatic ligaments are relaxed, producing the characteristic hollow appearance of the sacral region. In some animals, movement of the hindquarters produces a 'crepitus'-like sound, probably due to relaxation of the interosseous ligaments. In addition to these changes there appears in some cases to be a lack of tone in the musculature of the soft organs, which on a few occasions has led to prolapse of the vagina and rectum.



The mechanism underlying these changes is not known.

The part these changes play in the production of fractures is difficult to estimate; in some animals they produce slight disturbances in equilibrium and an unsteady gait, and it would seem probable that the alterations may impair the efficiency of the natural shock-absorbing mechanisms of the pelvis.

#### SUMMARY

Trauma from persistent coital mimicry was responsible for the fractures with the changes in pelvic morphology as a probable predisposing factor. There was no evidence of bone rarefaction.

I am greatly indebted to Prof. G. F. Boddie for his report on the radiographs. I wish to thank Miss H. M. Brnee for taking the X-ray photographs, and Dr K. M. Henry for the calcium and phosphorus analyses. My thanks are also due to Mr R. W. Hill, of the Ministry of Food, for granting permission to obtain the bones and for making the necessary arrangements at the various slaughterhouses.

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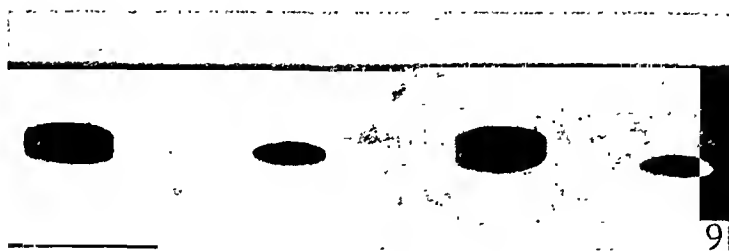
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#### EXPLANATION OF PLATE 1

FIG. 8. Section of whole udder from heifer HL 6. Maximum daily yield  $17\frac{1}{2}$  lb.

FIG. 9. Largest and smallest tablets from implant of 50 mg. tablets removed from cow CL 6, after 51 days *in situ*. Combined weight of large tablets=82.1 mg. Combined weight of small tablets=19.8 mg.

FIG. 10. *Top*: Sections of ovaries from an untreated heifer showing: right, a ripening follicle; left, a fully developed corpus luteum. *Bottom*: Sections of hypoplastic ovaries from a heifer slaughtered with  $6 \times 1000$  mg. hexoestrol tablets *in situ*. Three were implanted 41 days and three 17 days before slaughter. The absence of follicular development and of fresh luteal tissue is clearly demonstrated.  $\times 1.5$ .





# ARTIFICIAL INDUCTION OF LACTATION IN BOVINES BY ORAL ADMINISTRATION OF SYNTHETIC OESTROGENS

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In the preceding paper [Folley & Malpress, 1944] it has been shown that lactation can be induced in maiden heifers or dry cows by the subcutaneous implantation of tablets of diethylstilboestrol (hereinafter called stilboestrol) or hexoestrol. Dodds, Golberg, Lawson & Robinson [1938] found that stilboestrol was almost as active in the rat, by the vaginal-smear test, when given by mouth as when subcutaneously injected. It was therefore interesting to see whether lactation could be induced in cows and heifers by oral administration of synthetic oestrogens, since the feeding method possesses obvious practical advantages. Induction of lactation in goats by oral administration of stilboestrol was reported by Lewis & Turner [1942].

## MATERIAL AND METHODS

### *Experimental animals*

(a) A series of preliminary experiments was carried out on seven maiden heifers and one heifer (Regality) which had aborted early in pregnancy, each of which received individual treatment. Four of these (three Shorthorns and one pedigree Guernsey) belonged to this Institute; the other four were Ayrshires belonging to the Agricultural Research Council's Field Station, Compton.

(b) A large-scale experiment (Ilsley Dairy experiment) involving four treatments was carried out on four groups of eight Ayrshire maiden heifers, the majority of which were judged, from their state of dentition, to be under 2 years old at the start of the experiment. These animals were the property of the Agricultural Research Council and were maintained at Ilsley Dairy, specially made available for this experiment at Compton. The experiment was carried out under the aegis of the Agricultural Research Council's Conference on Lactation. In the summer months, except when feeding and being milked, the animals were loose in a concrete exercise yard; in the winter they were allowed out only in the day-time. They were fed a normal maintenance ration supplemented by a special dairy cake of pre-war standard, the supplement being increased with rising milk yield according to the usual practice. The four groups were made up to be as nearly as possible alike as regards age and body weight.

### *Experimental treatments*

The synthetic oestrogens used were diethylstilboestrol, *meso*-hexoestrol and dienoestrol (4:4'-dihydroxy- $\gamma$ : $\delta$ -diphenylhexadiene). The last was of particular interest, since the work of Barnes [1942] had indicated that dienoestrol was considerably more potent, when given by mouth, than stilboestrol in inhibiting lactation in women.

In six of the experiments on individual animals, oestrogen (hexoestrol or dienoestrol) was administered by incorporation of tablets containing soluble inert base into boluses made from a paste of ground cattle cake. Before use the boluses were dried in a steam oven. The hexoestrol tablets contained 50 mg. of hexoestrol and 450 mg. of inert base and the dienoestrol tablets 20 mg. of dienoestrol and 480 mg. of inert base. When it was desired to feed 25 mg. of hexoestrol daily, half tablets were used. In one experiment a mixture of one part of stilboestrol and two parts of stearic acid was fed. This was prepared by adding crystalline stilboestrol to molten stearic acid with vigorous mechanical stirring, which was continued while the mixture cooled to a solid mass. This was then ground in a mortar and the powder fed by sprinkling the required daily dose on the grain ration. It seemed possible that synthetic oestrogens might undergo destruction in the rumen by the action of micro-organisms. The object of the stearic-acid treatment was to coat the stilboestrol crystals with a substance which would be insoluble at the pH of the rumen, thereby affording protection against the micro-organisms, but which would be dissolved away in the small intestine. Dienoestrol was administered to one heifer in solution in the drinking water. The solution was prepared by slowly adding 8 mg. of dienoestrol, dissolved in a small volume of alcohol (2 ml.), to 6 l. of water, with constant stirring. On the average this animal drank 18 l. of solution containing 24 mg. of dienoestrol per day. By this method of administration, first used by Noble [1938] in the rat, it was hoped to secure more or less continuous absorption of oestrogen, with the possibility of eliciting the desired physiological effect with smaller doses than if administered twice daily in the food in the ordinary way. The treatment received by each animal used in these experiments is given in Table 1.

Table 1. *Details of treatment of heifers fed synthetic oestrogens (preliminary experiments)*

Heifer	Breed	Treatment	Approximate total dose of oestrogen administered g.	Remarks
Regality	Ayrshire	25 mg. of hexoestrol daily for 15 weeks, followed by 100 mg. of hexoestrol daily for 7 weeks	7.5	—
Gwen	Ayrshire	50 mg. of hexoestrol daily for 15 weeks	5.3	—
RC 118	Ayrshire	100 mg. of hexoestrol daily for 20½ weeks	14.4	—
Duehess	Ayrshire	200 mg. of hexoestrol daily for 13 weeks	18.2	—
Moth	Shorthorn	50 mg. of hexoestrol daily for 8 weeks, followed by 25 mg. of hexoestrol daily for 2½ weeks, and then by 100 mg. of hexoestrol daily for 12 weeks	(3.2) + 8.4	First 10½ weeks of treatment on low doses were ineffective
Cobweb	Shorthorn	20 mg. of dienoestrol daily for 8 weeks, followed by 40 mg. of dienoestrol daily for 15 weeks	(1.1) + 4.2	First 8 weeks of treatment on low doses were ineffective
Peasblossom	Shorthorn	24 mg. of dienoestrol daily in the drinking water for 21 weeks	3.5	—
Blackberry	Guernsey	50 mg. of diethylstilboestrol + 100 mg. of stearic acid daily for 9½ weeks	3.3	—

In the Ilsey Dairy experiment, two substances, stilboestrol and dienoestrol, were compared at two dose levels. The oestrogen was incorporated into cattle cubes by dropping 0.25 ml. of an alcoholic solution containing 100 mg./ml. of oestrogen on to each cube from a syringe. This volume of solution was completely absorbed by the cube. The alcohol was then evaporated by drying in the air oven. Determinations of the synthetic oestrogen content of treated cubes, selected at random, made in Prof. E. C. Dodds's laboratory, resulted in theoretical recovery of the added oestrogen. The required daily dose was equally divided between feeds given in the morning and afternoon, the necessary number of oestrogenic cubes being added to the normal ration. One or two heifers refused to eat the cubes and were force-fed by the herdsman.

Table 2. *Details of treatment of heifers fed synthetic oestrogens (Ilsey Dairy experiment)*

Group	Individual nos. of heifers in group	Treatment	Total amount of oestrogen administered to each animal g.
1	11-18	200 mg. of diethylstilboestrol daily for 23 weeks	32.2
2	21-24	50 mg. of diethylstilboestrol daily for 23 weeks	8.1
	25-28	50 mg. of diethylstilboestrol daily for 10 weeks, followed by 100 mg. of diethylstilboestrol daily for 13 weeks	12.6
3	31-38	200 mg. of dienoestrol daily for 23 weeks, except nos. 34 and 36, the feeding of which was stopped at the 22nd and 19th weeks respectively on account of vaginal prolapse	32.2 30.8 (no. 34) 26.6 (no. 36)
4	41-48	50 mg. of dienoestrol daily for 10 weeks, followed by 300 mg. of diethylstilboestrol daily for 8 weeks	20.3

The treatments given to these groups are set forth in Table 2. Though it was originally intended not to make any changes during the course of the experiment, the fact that the majority of the animals in groups 2 and 4 failed to respond afforded an opportunity in certain cases to study the effects of increased dosages.

#### *Udder preparation*

The udder was obtained from one of the heifers in the Ilsey Dairy experiment (no. 36) which was slaughtered during lactation (see below) and fixed and stained as described in the preceding paper [Folley & Malpress, 1944].

### RESULTS

#### *Induction of lactation*

*Preliminary experiments.* Lactation curves for the four Ayrshire heifers fed hexoestrol at various levels are given in Fig. 1. All animals secreted measurable amounts of fluid by the third week after the start of feeding. In these four experiments there was no strict relationship between the daily dose and the response, though the animal receiving the highest dose responded best, i.e. her yield showed the greatest rate of increase and reached the highest peak, while the animal receiving the smallest dose gave the least response. It is noteworthy that when the daily dose given to the latter was quadrupled, after 15 weeks of almost ineffectual treatment, the response was improved very little if at all. This may mean either that the inherent capacity of this animal to respond to the treatment was very low, or that

previous prolonged treatment with an ineffective dose of oestrogen prevents subsequent response to a higher dose. The latter alternative would seem to be contra-indicated by the results with the heifer Moth, and other results in the Ilsey Dairy experiment to be considered below.

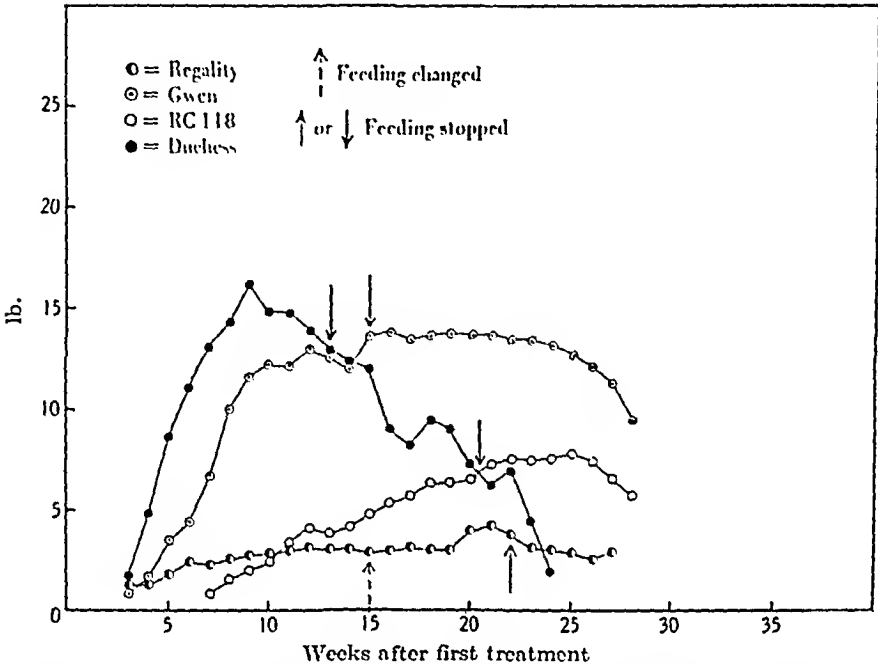


FIG. 1. Lactation curves for heifers fed hexoestrol. (Mean daily yields for 7-day periods.) Regality: 25 mg. of hexoestrol daily for 15 weeks; 100 mg. of hexoestrol daily for 7 weeks. Gwen: 50 mg. of hexoestrol daily for 15 weeks. RC 118: 100 mg. of hexoestrol daily for 20½ weeks. Duchess: 200 mg. of hexoestrol daily for 13 weeks.

In this series of experiments there was no suggestion of an increase in yield following cessation of feeding, though in all cases feeding was not stopped until some time after the maximum yield had been reached. The total amounts of milk secreted by these animals are given in Table 3.

Table 3. *Details of milk yields of heifers fed synthetic oestrogens (preliminary experiments)*

Heifer	Duration of treatment days	Duration of recorded lactation days	Maximum daily yield* lb.	Total recorded yield lb.	Yield when recording stopped* lb.	Remarks
Regality	164	175	4½	501	3	—
Gwen	105	182	13½	1978	9½	—
RC 118	143	154	7½	803	5½	—
Duchess	91	154	16½	1497	2	—
Moth	(73) + 84	280	18	3689	15	First 73 days of treatment on low dose were ineffective
Cobweb	(56) + 105	189	11	1483	8½	First 56 days of treatment on low dose were ineffective
Peasblossom	147	224	11½	1774	6½	—
Blackberry	66	189	13	1676	9	—

\* Mean for 7-day period.

Fig. 2 shows the lactation curves for the three Shorthorn heifers and one Guernsey heifer given various treatments. The response of the animal (Moth) which received 100 mg. of hexoestrol daily after 10½ weeks of ineffective treatment with a lower dose was roughly equal to that of Gwen, the Ayrshire heifer receiving 50 mg. daily. In this case, however, the yield rose slowly for some 11 weeks following cessation of treatment to a peak yield of 17 lb. daily and thereafter never fell below 12 lb. for a further 10 weeks when 400 mg. of stilboestrol dissolved in 10 ml. sesame oil was subcutaneously injected. The object of this treatment was to see whether the yield could thereby be further increased as was successfully accomplished by Walker & Stanley [1941] by periodical injections of diethylstilboestrol dipropionate in a heifer brought into artificial lactation.

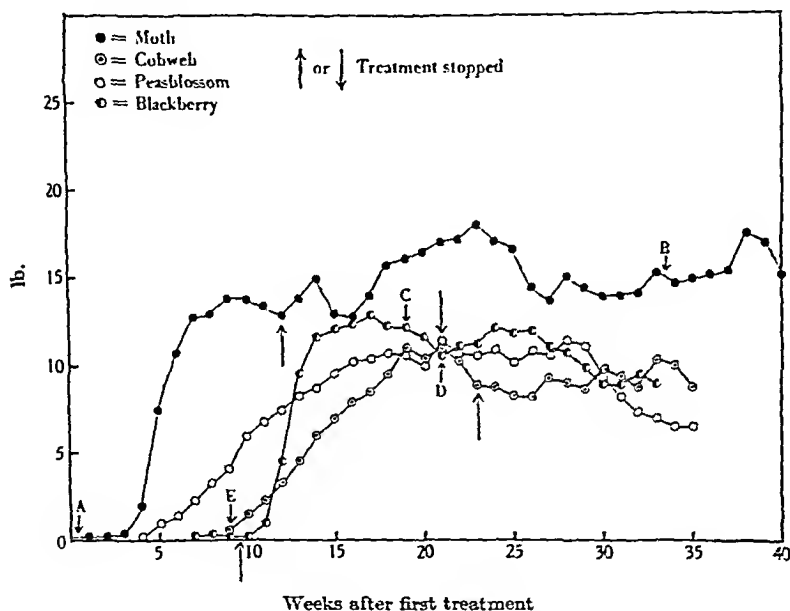


FIG. 2. Lactation curves for heifers receiving synthetic oestrogens in the food or drinking water. (Mean daily yields for 7-day periods.) Moth: 100 mg. of hexoestrol daily for 12 weeks. A, for earlier treatment see text; B, 400 mg. of diethylstilboestrol in 10 ml. sesame oil injected. Blackberry: 50 mg. of diethylstilboestrol daily for 9½ weeks fed as stearic acid—diethylstilboestrol mixture. C, D, similar treatment from 19th to 21st week. Peasblossom: 24 mg. of dienioestrol daily for 21 weeks dissolved in drinking water. Cobweb: 20 mg. of dienioestrol daily for 8 weeks; 40 mg. of dienioestrol daily for 15 weeks. E, aborted (9 weeks).

The heifer receiving 20 mg. of dienioestrol daily gave only small quantities of secretion after 8 weeks of treatment. The dose was then doubled, 8 days after which she aborted a foetus which was estimated from the crown-rump length (24 cm.) to be 3–4 months old. The abortion was quickly followed by a steady increase in milk yield but at a rather slow rate. The maximum yield was in the neighbourhood of 10 lb. daily. In the heifer receiving dienioestrol in aqueous solution, milk secretion started 4 weeks after the beginning of treatment. The yield thereafter rose steadily, but rather slowly, to a maximum of about 10 lb. daily and remained at that level



for 9 weeks after stopping treatment. The results of this one experiment gave no indication that the administration of oestrogen in the drinking water possessed any advantages over twice daily administration of crystalline material incorporated into the food, as regards the total amount of oestrogen required to induce lactation. Further, this procedure was manifestly more complicated in practice. For these reasons no further experiments along these lines were carried out.

The case of Blackberry, the Guernsey heifer which received the stilboestrol-stearic acid mixture, is of interest, in that after  $9\frac{1}{2}$  weeks' treatment she gave only small amounts of secretion, but that when feeding was stopped the milk yield rapidly increased to a peak of about 12 lb. daily; thereafter the yield slowly declined despite a subsequent period, lasting 2 weeks, of similar treatment with the same oestrogenic mixture.

The total milk yields of these four heifers are also given in Table 3.

*Ilseley Dairy experiment.* Lactation curves for those animals in this experiment which gave a positive response to treatment are shown in Figs. 3-6 and details of maximum and total yields in Table 4.

Table 4. *Details of milk yields of heifers fed synthetic oestrogens (Ilseley Dairy experiment)*

Group	Heifer	Duration of treatment days	Duration of recorded lactation days	Maximum daily yield* lb.	Total recorded yield lb.	Yield when recording stopped* lb.	Remarks
1	13	161	217	$16\frac{1}{2}$	2296	$13\frac{1}{2}$	Yielding 6 lb. daily after a further 15 weeks. Estimated total yield: 3300 lb. in 322 days
	14	161	217	$11\frac{1}{2}$	1802	9	—
	15	161	210	$6\frac{1}{2}$	869	$3\frac{1}{2}$	—
	18	161	210	$9\frac{1}{2}$	1041	$7\frac{1}{2}$	—
2	22	161	161	9	873	$7\frac{1}{2}$	—
	26	161	161	$10\frac{1}{2}$	1063	$8\frac{1}{2}$	—
	27	161	119	$2\frac{1}{2}$	133	$\frac{1}{2}$	—
3	31	161	154	19	2349	16	Yielding 11 lb. daily after a further 15 weeks. Estimated total yield: 3760 lb. in 259 days
	32	161	119	$9\frac{1}{2}$	729	$8\frac{1}{2}$	—
	33	161	210	6	791	$4\frac{1}{2}$	—
	35	161	175	$2\frac{1}{2}$	230	$\frac{3}{4}$	—
	36	133	175	$8\frac{3}{4}$	878	7	—
	38	161	105	$12\frac{1}{2}$	776	$9\frac{1}{2}$	—
4	41	126	175	$3\frac{1}{2}$	431	$2\frac{1}{2}$	—
	43	126	154	$4\frac{1}{2}$	417	$3\frac{3}{4}$	—
	44	126	147	$11\frac{1}{2}$	1116	11	Yielding 9 lb. daily after a further 15 weeks. Estimated total yield: 2150 lb. in 252 days
	45	126	147	$2\frac{1}{2}$	216	$\frac{3}{4}$	—
	47	126	161	11	1201	9	—
	48	126	42	$5\frac{3}{4}$	127	$5\frac{1}{2}$	—

\* Mean for 7-day period.

As regards the response to the higher dose of each of the two oestrogens, reference to Figs. 3 and 5 shows that of the eight heifers receiving stilboestrol only four came into lactation, while positive responses were given by six of the eight receiving

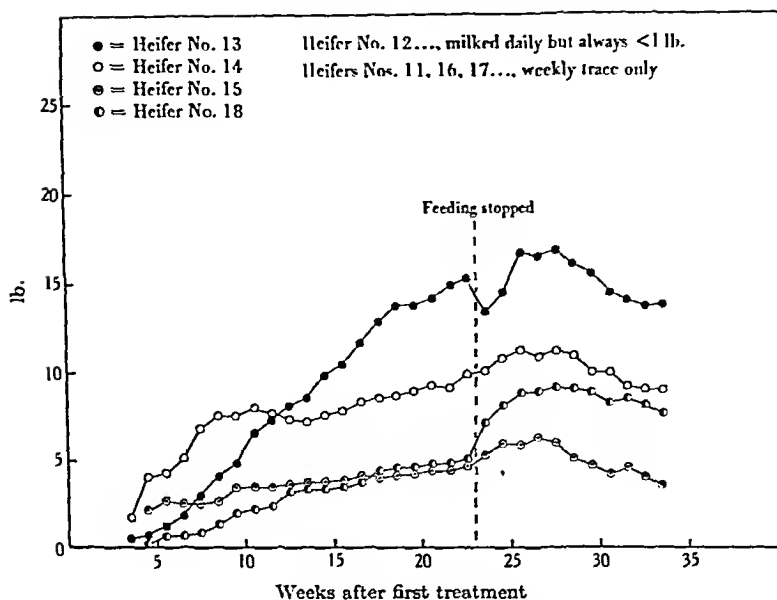


FIG. 3. Lactation curves for heifers fed 200 mg. of diethylstilboestrol daily. (Mean daily yields for 7-day periods.)

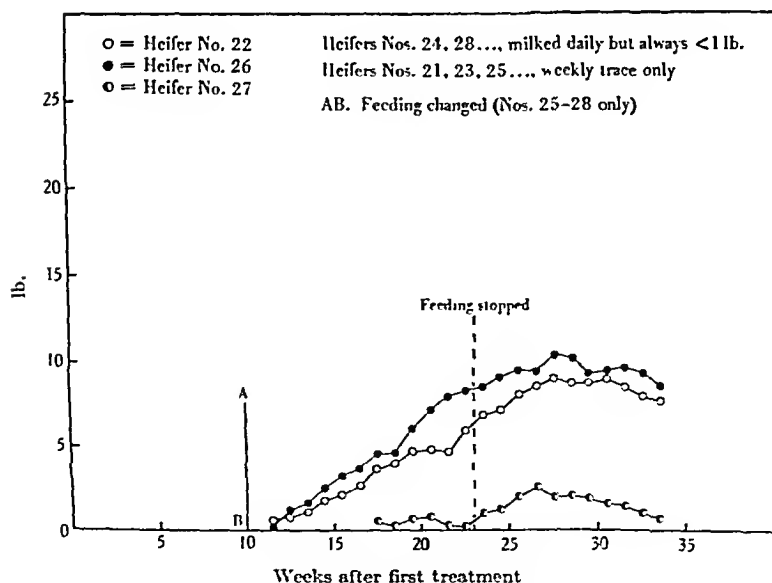


FIG. 4. Lactation curves for heifers fed 50 mg. of diethylstilboestrol daily for 23 weeks (nos. 21-24), or 50 mg. of diethylstilboestrol daily for 10 weeks followed by 100 mg. of diethylstilboestrol daily for 13 weeks (nos. 25-28). (Mean daily yields for 7-day periods.)

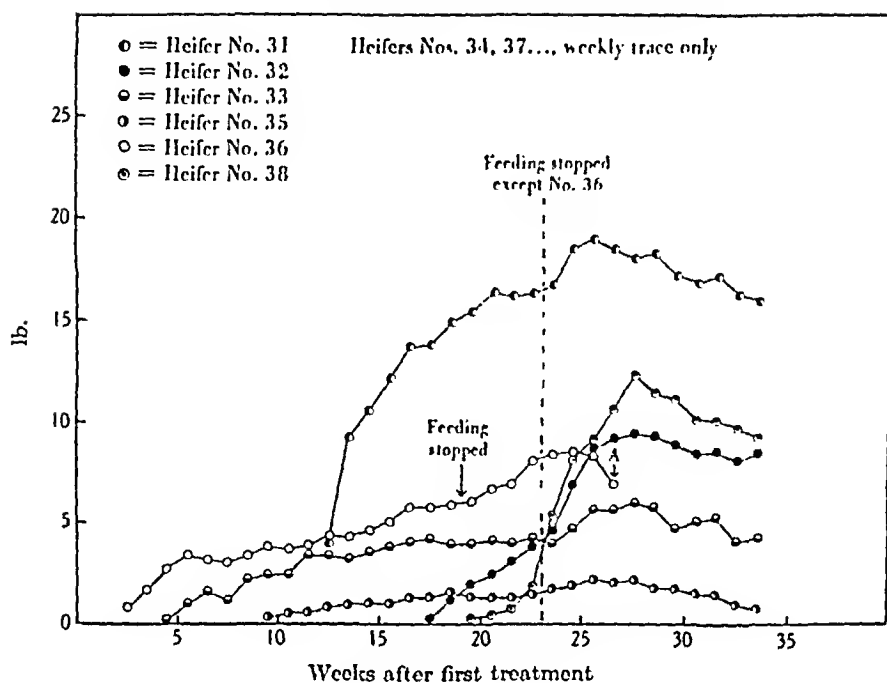


FIG. 5. Lactation curves for heifers fed 200 mg. of dioenoestrol daily. (Mean daily yields for 7-day periods.) A, heifer 36 slaughtered.

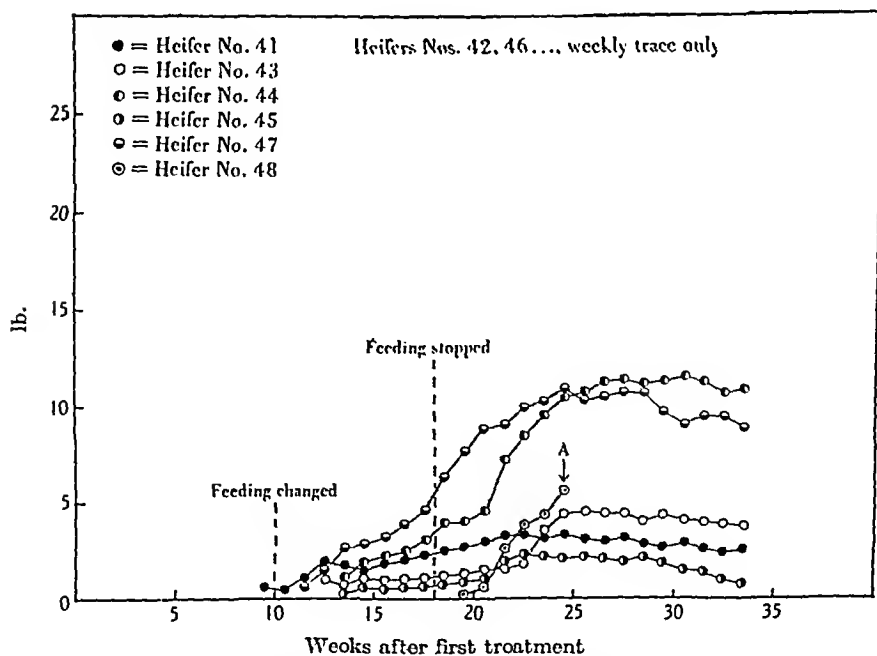


FIG. 6. Lactation curves for heifers fed 50 mg. of dioenoestrol daily for 10 weeks and 300 mg. of diethylstilboestrol daily for 8 weeks. (Mean daily yields for 7-day periods.) A, heifer 48 slaughtered.

dienoestrol. After induction of lactation, with three exceptions referred to below, the yields of the positive animals of both groups increased rather slowly, the rates of increase being appreciably less than in the more successful experiments involving tablet implantation [Folley & Malpress, 1944]; the maximum yields attained were also, on the whole, lower. Three of the animals receiving dienioestrol, however, showed rates of increase in milk yield which were markedly greater than those shown by the other responsive animals receiving either oestrogen. It is further noteworthy that in all three cases the onset of lactation was appreciably delayed, occurring in no. 31 after 12 weeks' treatment and in nos. 32 and 38 after 17 and 19 weeks respectively. The results permit the conclusion that at the higher level of dosage there was no appreciable difference between stilboestrol and dienioestrol as regards their efficacy in inducing mammary development and lactation.

For both substances, 200 mg. daily gave more successful results than 50 mg. daily. By the end of the tenth week of treatment, no heifer receiving 50 mg. of stilboestrol daily had come into lactation (Fig. 4), while one only of the heifers in the group receiving 50 mg. of dienioestrol was giving a slight amount of secretion (Fig. 6). On the other hand, by this time, four heifers fed 200 mg. of stilboestrol daily were giving yields varying from 2 to 7 lb. daily and three receiving the higher dose of dienioestrol had begun to lactate.

Increasing the dose of stilboestrol from 50 mg. daily to 100 mg. from the tenth week onward hardly improved matters. Of the four heifers in group 2 which underwent this change of treatment, one (no. 26) slowly came into lactation shortly after the dose was increased and another (no. 27) began giving a small amount of secretion 7 weeks later. However, one of the four heifers (no. 22) whose treatment was not changed also began to lactate at the same time as no. 26 and the lactation curves of the two heifers thereafter ran fairly parallel, so that it is not possible to conclude that the positive result with no. 26 was due to the increase in the dose.

The substitution, at the tenth week, of a daily dose of 300 mg. of stilboestrol for the 50 mg. of dienioestrol fed daily to the heifers in group 4 did produce more positive results. After the change, six of the heifers came into milk, one of them (no. 48) shortly after the treatment was stopped, but, at the best, the peak yields were unsatisfactory.

In a number of cases, nos. 15 and 18 (Fig. 3), no. 27 (Fig. 4), nos. 31, 32, 33 (Fig. 5) and no. 47 (Fig. 6), the rate of increase in milk yield rose to some extent immediately after the cessation of feeding. It may also be noted that the lactation curves of nos. 43, 44, 45 and 48 (Fig. 6) underwent a delayed rise some weeks after feeding was stopped. In other cases, the course of the lactation curve was unaffected when the treatment ceased.

A longitudinal section through the right half of the udder of no. 36, a heifer which received 200 mg. of dienioestrol daily and which was slaughtered (see below) when giving just over 6 lb. daily, is shown in Plate 1, fig. 7. It will be seen that an extensive alveolar system is present.

#### *Effects of the treatment on the reproductive organs*

Throughout the Ilsey Dairy experiment, the ovaries and uteri of all the heifers were examined by rectal palpation at intervals of 2-8 weeks. In all there were twelve examinations extending over a period of a year, the first being made before

the treatment started. They were carried out by Mr D. N. Spriggs, M.R.C.V.S., whose report is paraphrased below.

*Ovaries.* Within 2 months of the beginning of oestrogen administration the ovaries of every animal had become inactive, the acyclic phase being noted earliest in groups 1 and 3. In the majority of the animals comprising these two groups, ovarian inactivity was noted within 2 weeks of the beginning of treatment.

Over a period of some 2 months from the cessation of treatment, the ovaries of approximately 25 % of animals in all groups contained small cysts (or possibly large follicles). In many cases, corpora lutea were present in the ovaries at this time, indicating a return to normal function.

*Subsequent reproductive history.* Service of approximately half of the heifers, those in which the ovaries were considered normal and in which the oestrous cycle had been re-established, began about 3 months after the cessation of feeding. The remainder were found to be normal and hence ready for service about 1 month later. The majority of the heifers became pregnant to the first service. At the time of writing, pregnancy diagnosis by palpation of the uterus has established that, of the remaining thirty heifers (see below), twenty-five are definitely pregnant and two possibly pregnant.

*Nymphomania.* Symptoms of nymphomania were not marked in this experiment. Only one case of persistent oestrus was observed; in most animals irregularly intermittent oestrous behaviour was displayed. No animal suffered fracture of the pelvis or femur, though during the period of feeding, four heifers (nos. 34, 36, 38 and 48) showed periodic prolapse of the vagina. Nos. 36 and 48 in addition showed rectal prolapse. In these two animals the condition persisted after cessation of treatment and finally advanced to a climax which necessitated their slaughter.

#### *Effects of oestrogenic treatment on the health and condition of the animals*

Observations were made at intervals throughout the Ilsey Dairy experiment on the general health and bodily condition of the animals. These observations, together with measurements of the pelvic bones, were made by Mr S. L. Green on whose report this section is based. Live weights were recorded before the treatment began and on two occasions during treatment.

Most of the animals began the experiment footsore and generally lacking in 'body tone' and 'bloom' as a result of a railway journey lasting 4 days. In the earlier part of the feeding period all animals improved in 'condition' (using the word 'condition' in the special sense in which it is used in animal husbandry) and increased in weight. Throughout the experiment there was no evidence that the treatment exerted any adverse effects on general health or bodily 'condition'.

In all except three animals (nos. 21, 22 and 47) treatment was accompanied by some elevation of the tail-head. This disfiguration was still noticeable in most of the heifers 6 months after the cessation of treatment. On the whole, however, the degree of elevation of the tail-head encountered in this experiment was less marked than in experiments involving tablet implantation [Folley & Malpress, 1944], a fact which probably accounts for the absence of pelvic fractures among the fed animals.

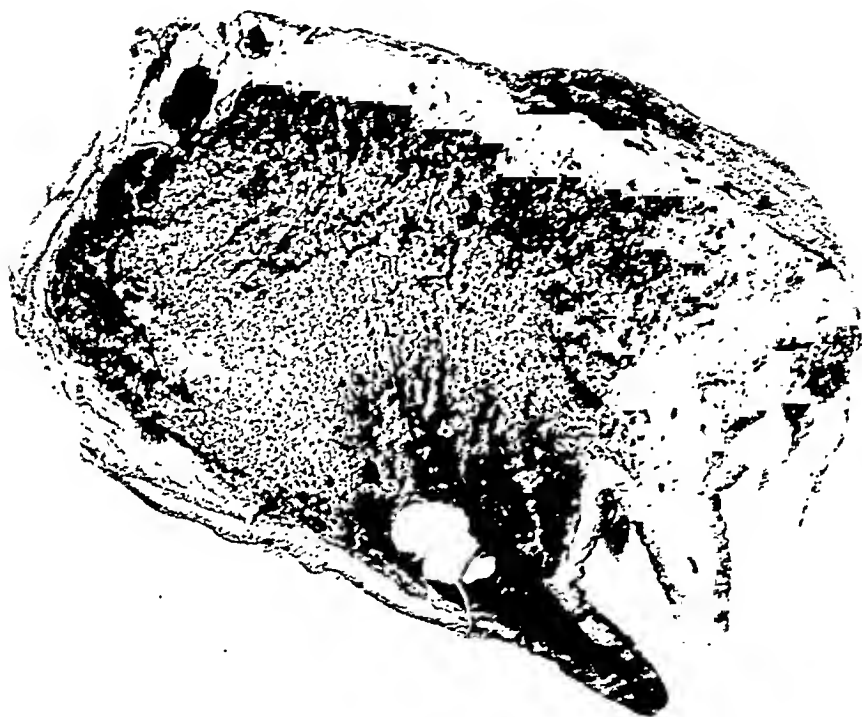


FIG. 7. Section of whole udder from heifer 36. Maximum daily yield  $8\frac{1}{2}$  lb.



## DISCUSSION

From the results of the preliminary experiments it appeared likely that a daily oral dose of 50–100 mg. of hexoestrol, given over a period of about 10 weeks, i.e. a total dose of 3.5–7.0 g. of synthetic oestrogen, would give results comparable with those obtained by tablet implantation. An experiment carried out on a larger scale therefore appeared to be justified.

Unfortunately, the results of the experiment on comparable groups of heifers did not bear out the promise of the preliminary experiments. Only nineteen (59%) of the thirty-two heifers used gave more than 1 lb. of milk at any milking, and in practically every case the rate of increase in milk yield and the maximum yield attained was disappointingly small. The individual variations in response which were a feature of the tablet implantation experiments [Folley & Malpress, 1944] were evident in this experiment also, but the general plane of response was considerably lower.

It might be argued that 50 mg. daily may have been too low a dose of either oestrogen and 200 mg. too high. It is to be expected that, beyond a certain point, as the amount of oestrogen reaching the circulation increases, the lactational response would fall off, first because excessive doses of oestrogen result in diminished or abnormal mammary duct development [Gardner, 1941], and second because high doses of oestrogen tend to exert an inhibitory effect on lactation [Folley, 1941]. The effects of the removal of such an inhibition were observed in the experiments on tablet implantation. The results given by those animals in group 2, whose daily dose was eventually raised from 50 to 100 mg. of stilboestrol, do not support this view however, since the response to 100 mg. of stilboestrol was no better than the response to 50 mg. The Ilsley Dairy experiment, as finally carried out, allowed some sort of a comparison between the responses to 50, 100, 200 and 300 mg. of stilboestrol, and the indications are that the optimum dose was in the neighbourhood of 200 mg. daily. Since this experiment afforded no evidence that in cows, dienoestrol is more active by the oral route than stilboestrol, the same conclusion probably applies to dienoestrol.

In attempting to put forward an explanation for the discrepancy between the results of the preliminary experiments and the Ilsley Dairy experiment, the possibility that the disappointing results in the latter experiment might have been due to the use of heifers that were too immature to respond satisfactorily is worth consideration, granted the likely assumption that young heifers respond less well to oestrogenic treatment than fully mature animals. Unfortunately, since the ages of the animals used in the Ilsley Dairy experiment were not known with certainty, no exact analysis of the results on this basis is possible, though it is fairly certain that most of them were under 2 years old at the start of the experiment, i.e. they may well have been too young to respond satisfactorily. The possibility that the efficiency of utilization of the ingested oestrogen was lower in the Ilsley Dairy experiment than in the preliminary experiments, possibly because of dietary differences, would be worth consideration were it not for the fact that even at dose levels that must be considered very high (200 and 300 mg. daily) the response of the heifers in the group experiment was poor. If differences in utilization efficiency were involved, it should



have been possible to increase the amount utilized to the level necessary for a good response by sufficiently increasing the intake. To explain the findings on the basis under discussion it would be necessary to assume that only a certain amount of oestrogen could be utilized no matter how much is fed, and that this saturation value was much less for the heifers in the Ilsley Dairy experiment than for those used in the preliminary experiments.

It is of interest to attempt to compare the lactational responses obtained by the feeding and tablet implantation methods, in relation to the doses of oestrogen employed. No more than a very rough comparison between the present results and the results with tablets described previously is possible, because so many different types and degrees of response were encountered in the two sets of experiments.

In the implantation experiments the mean daily absorption rates from 2.5 g. implants were approximately 12 mg. for hexoestrol and 14 mg. for stilboestrol. Allowing for ghost formation it may be concluded that satisfactory lactational responses in many cases resulted from the entry of roughly 15–20 mg. of oestrogen into the circulation daily. Now in the Ilsley Dairy feeding experiment, about 200 mg. daily of either stilboestrol or dienestrol were required to evoke positive responses. It would therefore appear that not more than about 10% of this daily dose entered the circulation. In point of fact, the available evidence indicates that the utilization was considerably less, since only about half of the heifers which received 200 mg. of oestrogen daily came into milk and in these the onset of lactation was much slower than in the tablet-implanted animals. It is impossible to be quantitative, but it is safe to say that the fed animals were at least four times as slow. If the speed of onset of artificial lactation is, over a limited range, roughly proportional to the daily dose of oestrogen, it would thus appear that of the 200 mg. of oestrogen ingested daily by these heifers only an amount of the order of 5 mg. entered the circulation.

The factor of age may further complicate this comparison, if the degree of maturity of the test animal affects the lactation response. The heifers used in the implantation experiments were in most cases at least 3 years old, i.e. considerably more mature than most of the animals used in the Ilsley Dairy experiment. The animals used in the preliminary feeding experiment were probably more comparable in this respect with those implanted with tablets. Three of these animals gave responses comparable with those given by implanted animals when fed 50, 100 and 200 mg. of hexoestrol daily, of which amounts hardly more than 20 mg. daily can have been absorbed.

The general validity of these rough estimates is supported by quite independent arguments based on the fact that the nymphomaniac syndrome was a constant and strongly marked feature of the implantation experiments while it was much less marked in the feeding experiments, even in animals receiving 200 mg. of oestrogen daily. This would indicate that in the latter animals considerably less than 20 mg. of oestrogen entered the circulation daily.

It would thus appear that a large proportion of synthetic oestrogen orally administered to bovines fails to enter the systemic circulation. It seems likely that this is due to destruction of oestrogen in the rumen while not excluding the alternative possibilities that in this species, for unexplained reasons, much of it may escape absorption in the intestine and pass out unchanged into the faeces or that being well absorbed into the portal circulation a considerable fraction is inactivated in the liver.

If an appreciable proportion is unabsorbed, the cases of vaginal and rectal prolapse, which occurred in the feeding experiments, may have been due to the local action, on the sensitive mucosa, of oestrogen reaching this area in the faeces.

The low efficiency of utilization of orally administered oestrogen constitutes a most definite disadvantage on economic grounds which offsets the two great advantages of oral administration, namely, simplicity in practice and flexibility allowing of day-to-day control of oestrogen intake. This last is an especially valuable attribute in the case of a treatment for which it is hard to postulate standard conditions, but where each animal is best considered individually. We feel that in the light of the partially successful results obtained in these experiments, further research directed towards increasing the efficiency of utilization of synthetic oestrogens, orally administered to bovines, is desirable.

#### SUMMARY

1. A preliminary experiment in which eight heifers were given daily doses of hexoestrol or dienoestrol in their food or drinking water indicated that lactation could be satisfactorily induced in this way.

2. A further experiment involving thirty-two heifers, in which diethylstilboestrol or dienoestrol was fed daily, gave less encouraging results, only nineteen of the animals being brought into lactation and the general plane of response being markedly lower.

3. Variability of the response in all its quantitative aspects was a feature of these experiments.

4. The yields generally were inferior to those given in the implantation experiments reported in the previous paper, and far more oestrogen was needed to evoke responses.

5. The general 'condition' (using the word 'condition' in the special sense in which it is used in animal husbandry) of the animals suffered in no way from the treatment, and nymphomaniac effects were not pronounced. No pelvic fractures were caused, but two serious cases of vaginal and rectal prolapse were encountered.

6. Thirty of the animals used in the main experiment have since been served and twenty-five have become pregnant, in many cases to the first service.

7. It is estimated that in the bovine, the oral administration of synthetic oestrogens results in the utilization of less than 10% of the dose given. The special problems presented by rumen activity and incomplete absorption from the alimentary canal are considered.

8. The extent to which the factor of age may influence the response is also discussed.

This work was carried out during the tenure by F. H. M. of a research grant from the Agricultural Research Council, to whom we are also greatly indebted for the provision of the heifers used in the main experiment. Our best thanks are due to Dr W. S. Gordon for providing accommodation for these animals and indispensable facilities for the experiment. The late Mr W. G. Dunkin kindly gave us access to four of the animals used in the preliminary experiments. For the care of the above animals we are grateful to Messrs Maltby and Cook. It is a pleasure also to express our thanks to Messrs D. N. Spriggs and S. L. Green for expert co-operation in the

Ilsley Dairy experiment; to Prof. E. C. Dodds, F.R.S., for suggesting the experiment involving administration of oestrogen in the drinking water, and for determinations of the oestrogen content of impregnated cattle cubes, and to Mr R. W. Hill of the Ministry of Food for enabling us to recover the udder of heifer 36. Some of the dienocestrol used in the preliminary work was kindly provided by Messrs Glaxo Laboratories Ltd.

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*Note added 14 March 1944.* The subsequent history of the thirty-two heifers used in the Ilsley Dairy experiment may now be summarized as follows.

Twenty-three calved normally, producing twenty-four calves, two of which died at birth (they comprise eleven males and eleven females); two are due to calve shortly; three were sold as barren; two were slaughtered during the experiment; two were slaughtered later owing to actinomycosis, at least one of these was pregnant. The heifers which have calved are milking satisfactorily, the best performers, on the whole, being those which responded best to oestrogen. Elevation of the tail head is still noticeable and calving tended to be a little difficult in animals showing pronounced pelvic changes.

# THE CHEMICAL COMPOSITION OF BOVINE MAMMARY SECRETIONS INDUCED BY THE SUBCUTANEOUS IMPLANTATION OR ORAL ADMINISTRATION OF SYNTHETIC OESTROGENS

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(Received 17 December 1943)

The only study so far recorded of the chemical composition of milk produced by ruminants artificially brought into lactation by treatment with synthetic oestrogens, carried out by Folley, Scott Watson & Bottomley [1941*b*], showed that the milk from virgin goats receiving regular udder injections with 1% diethylstilboestrol ointment underwent changes qualitatively similar to those occurring after parturition during the transition from colostrum to normal milk. These changes, which took place more gradually in oestrogen-treated than in normal parturient animals, follow the initial secretion of fluids characterized by high fat and non-fatty solid (S.N.F.) contents, coupled with a high total nitrogen value, an abnormally low casein number and a high globulin number. No parallel study has hitherto been made upon artificially induced mammary secretions in the bovine, though Folley [1936] investigated the effect of natural oestrogens on milk composition in established lactation in this species and observed increases in fat and S.N.F. values. Similar results were obtained with synthetic oestrogens [Folley *et al.* 1941*a*].

## EXPERIMENTAL

Changes in milk composition were recorded for seven heifers receiving synthetic oestrogen implants [Folley & Malpress, 1944*a*] and for sixteen heifers receiving synthetic oestrogen orally [Folley & Malpress, 1944*b*], the animals being chosen at random but being representative of the various subgroups participating in these experiments. The uniform picture presented by the results obtained makes it unnecessary to give all the analyses in detail; consequently only typical results from the main (small tablet) implantation experiment and from the large-scale (Ilseley Dairy) feeding experiment are reported here in full. Essentially the same progressive changes in chemical composition have been found in the milk samples from the heifers used in individual feeding experiments and from the only heifer implanted with a large hexoestrol tablet whose milk was examined.

Composite milk samples for analysis were prepared by mixing samples taken at the morning and afternoon milkings in the ratio of the respective yields. In cases where the daily yield was less than 5 lb. the animals were milked once daily and single samples used accordingly. All samples were stored overnight in the ice-chest, the various milk solutions required for the analyses being prepared the next morning.

With the exception of two heifers (HL 12 and no. 26) the first sample analysed in each individual series was the first measurable sample withdrawn from that animal.

Analytical methods were as follows. Total solids were determined by Golding's [1934] method and fat percentage by the Gerber method, non-fatty solids being given by difference. For determinations of nitrogen distribution, non-casein, globulin and non-protein fractions were prepared by methods essentially similar to those of Rowland [1938], nitrogen being estimated by the micro-Kjeldahl method. On account of the time-consuming nature of the determinations it was only possible to estimate globulin nitrogen in a few cases. Lactose was determined by the method of Blackwood [1934] and chloride by Davies's [1938] method. For phosphatase (phosphomonoesterase A<sub>1</sub> [Folley & Kay, 1936a]) the method of King & Armstrong [1934] was used.

### RESULTS

The illustrative results are given in Tables 1 and 2. The earliest secretions were invariably colostrum in general character and were characterized by high S.N.F. and low fat percentages. In the two parturient cows studied by Elsdon [1934], the colostrum fat percentage rose to a maximum before stabilizing itself at a lower, more normal, value. Evidence of a similar maximum was shown by series of samples taken from heifers HL 1, HL 2 and HL 11, used in the implantation experiment. This effect was not observed in samples from heifers in the feeding experiment, possibly because of the low rate of increase in lactation in these animals.

The total nitrogen values and the nitrogen distribution figures were at first typical of colostrum (compare figures for the nitrogenous constituents of normal bovine colostrum given by Engel & Schlag [1924] and Crowther & Raistrick [1916]). Since, as lactation advanced, changes in the non-protein nitrogen were small, the 'soluble' protein (albumin + globulin), of which fraction globulin forms the major proportion in normal colostrum [Crowther & Raistrick, 1916], necessarily showed a complementary relationship with casein. Though globulin determinations were not made in any of the cases recorded in Tables 1 and 2, it may safely be concluded that had they been carried out, high globulin numbers would have been found in the early stages, since casein numbers of less than 50 (limits for normal milk 76–80) were frequent. In cases where globulin determinations were made, globulin numbers above 30 (upper limit for normal milk is approximately 5) were found in the early stages of artificially induced lactations. For a short discussion of the significance of casein and globulin numbers in relation to normal milk and secretions of a colostrum type see Folley *et al.* [1941b].

As would be expected from a consideration of the osmotic equilibrium maintained by mammary secretions, the lactose and chloride concentrations varied in an opposite sense, lactose content increased with yield and both reached values characteristic of true milk at roughly the same rate as the other milk components. Phosphatase activity, measured in King-Armstrong units, was found to decrease precipitously and to reach normal values (< 100) very soon after secretion had begun. This is in general accord with the findings of Folley & Kay [1936b] for lactation in the normal cow.

### DISCUSSION

It is evident from the analyses reported here that the artificially induced secretions from maiden heifers have initially a colostrum nature, but their composition changes to that characteristic of normal milk with a rapidity dependent more upon the rate

Table 1. *Composition of mammary secretions from heifers implanted with small tablets of synthetic oestrogens*

Heifer	Treatment*	Days after implan- tation	Daily milk yield lb. oz.	Fat %	S.N.F. %	Total N mg. % 500-1000 2000	Casein N mg. % 385-462 680	Non- protein N mg. % 1.40	Casein† no.	Lactose %	Cl mg. %
—	Normal†	—	—	3.07	0.02	—	—	—	70-78	4.78	80-100
—	Colestrat†	—	—	—	—	—	—	—	34.0	2.10	163
III 1	290 × 25 mg. Hex.	10	1 0	1.05	11.40	952	474	55	49.8	3.18	182
		20	3 8	5.70	0.88	777	588	37	75.8	4.33	02
		42	13 0	4.25	9.39	603	408	30	77.0	4.84	40
		00	15 12	4.00	9.51	582	452	31	77.7	—	70
III 2	160 × 15 mg. Dues.	10	1 8	2.45	11.53	1227	855	50	60.7	3.06	120
		20	4 12	8.20	10.14	783	575	43	73.4	4.75	55
		42	16 8	4.90	9.54	622	400	30	75.4	4.05	45
		90	21 0	4.50	9.52	588	440	28	76.3	—	50
III 3	100 × 25 mg. Hex.	10	0 10	0.35	13.93	1018	747	01	46.2	3.70	180
		20	1 4	1.45	10.30	850	554	45	61.7	4.16	117
		42	12 0	4.40	9.01	576	427	37	74.2	4.04	58
		90	12 0	4.30	8.97	537	401	30	74.7	—	63
III 4	200 × 25 mg. Dues.	10	0 10	0.40	10.20	2115	751	48	35.5	1.80	70
		21	1 12	5.05	11.07	1074	615	41	57.3	2.82	—
		43	4 8	5.55	9.21	652	484	37	74.2	4.62	07
		50	8 8	4.10	9.14	575	445	35	77.4	4.02	70
		82	8 4	4.00	8.84	521	411	31	78.8	—	—
III 12	190 × 25 mg. Dues.	21	5 0	2.60	10.00	935	672	30	71.8	3.86	—
		43	14 0	4.30	9.74	631	490	20	77.7	5.16	140
		59	15 12	4.75	9.63	605	470	26	77.7	4.86	03
		82	13 12	4.10	9.26	585	461	28	78.8	—	—
III 15	160 × 15 mg. Hex.	10	1 6	3.15	8.92	1153	780	31	67.7	2.84	200
		20	5 0	5.20	9.98	795	564	36	76.0	4.32	—
		40	15 8	6.05	9.53	620	469	33	74.0	4.82	—

\* Hex. = hexoestrol; Dues. = diethylstilbestrol. For details of treatment see Folley &amp; Malpass [1944a].

† Quoted from Davies [1939].  
‡ Casein no. =  $\frac{\text{Casein N}}{\text{Total N}} \times 100$ .



36	14	0	1	2.80	11.80	1200	704	33.3	58.4	3.46	108	—
	21	1	3	3.50	10.00	758	555	32.4	73.2	4.24	109	83
	28	2	2	3.78	9.89	683	517	47.3	75.7	4.28	123	28
	42	3	1	4.23	9.80	620	408	32.8	75.5	4.02	100	30
	63	3	12	4.15	9.38	613	477	27.5	77.8	4.08	87	—
41	49	0	0	1.10	15.25	2129	718	47.8	35.1	1.38	200	252
	59	Tmco		—	—	980	495	27.9	50.2	2.78	215	63
	63	0	7	4.45	10.70	1011	570	33.5	50.2	3.56	163	130
	70	0	8	3.95	9.57	766	530	34.6	60.2	3.00	145	—
	77	0	8	5.20	9.47	685	401	30.6	67.3	4.36	120	61
	84	1	12	5.35	10.31	801	552	32.1	68.9	4.28	107	118
	91	1	12	5.50	9.00	672	493	28.0	73.1	—	—	113
	98	1	12	5.40	9.86	657	483	21.7	73.1	—	—	117
	112	2	0	6.20	9.65	626	470	20.5	75.1	—	—	—
43	84	1	13	5.20	11.50	1214	628	33.2	51.7	2.82	200	112
	91	0	12	5.75	9.34	991	501	22.5	72.5	—	—	32
	98	1	2	5.25	9.43	668	471	25.3	70.5	—	—	38
	112	0	15	6.15	9.44	636	457	20.9	71.9	—	—	—
	146	1	8	5.30	9.39	641	445	21.5	69.1	—	—	—
47	77	0	7	3.35	19.07	2639	1229	40.2	46.0	2.32	248	372
	84	1	4	5.53	10.70	974	614	45.4	63.0	3.48	155	48
	91	2	8	4.00	9.73	725	529	34.7	73.0	—	—	21
	98	3	4	4.70	9.46	628	470	20.7	75.8	—	—	—

† Casein no. =  $\frac{\text{Casein N}}{\text{Total N}} \times 100$ .

\* For details of treatment see Folloy & Malpress [1944b].



of increase of milk yield than the duration of oestrogen treatment. If the yield remains very low (e.g. in heifers 12 and 35) the abnormal values for the milk components persist; on the other hand, the maintenance of a steady yield of even  $\frac{1}{2}$  lb. daily (e.g. in heifers 18 and 41) clearly favours a slow trend towards normality. In general a daily yield of 5 lb., and often much less, could be regarded as a guarantee of normal composition.

Unlike the main milk constituents, phosphatase activity was unrelated to the amount of secretion, and whether the milk yield rose or fell after the first withdrawal the phosphatase unitage rapidly decreased (cf. heifers 12, 13 and 47). It would seem probable, therefore, that the very high initial values had some close connexion with the resting state of the gland or with the very early stages of the transition from a developmental to a secretory phase, rather than with the rate of production of milk *per se*, a supposition which receives support from the presence of greater concentrations of phosphatase in the non-lactating mammary glands of guinea-pigs, compared with their lactating glands [Folley & Kay, 1935].

The important question of the oestrogen content of the milk from oestrogen-treated animals is dealt with in a separate communication [Lawson, Stroud & Williams, 1944]. It is clear that in all respects pertaining to the main milk constituents, the milk obtained from oestrogen-stimulated mammary glands is of excellent quality and well-suited for human consumption.

#### SUMMARY

The mammary secretions produced by heifers artificially brought into lactation by treatment with synthetic oestrogens were at the outset colostral in nature, but the composition gradually changed to that characteristic of milk of good chemical quality at a rate roughly proportional to the rate of increase in yield. In general, a daily yield of 5 lb., sometimes much less, could be regarded as a guarantee of normal composition.

We are indebted to Dr S. J. Rowland for the chloride determinations and to Mr A. Wagstaff for the determinations of fat and total solids. The skilled technical assistance of Mr S. C. Watson and Miss D. Woolcott is gratefully acknowledged. This work was carried out during the tenure by F. H. M. of a research grant from the Agricultural Research Council.

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# EXPERIMENTS ON THE USE OF TABLETS CONTAINING 50 % HEXOESTROL FOR THE ARTIFICIAL INDUCTION OF LACTATION IN THE BOVINE

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*(Received 17 December 1943)*

The artificial induction of lactation in maiden heifers and dry cows by the subcutaneous implantation of tablets of diethylstilboestrol or hexoestrol has been described previously [Folley & Malpress, 1944*a*; Hammond, Jr. & Day, 1944]. In these experiments, when the animals came into milk, considerable proportions of the implants remained unabsorbed, and it was necessary to remove completely the remaining oestrogen after a suitable lapse of time.

The object of the experiments reported below was to investigate the possibility of using for this purpose tablets containing a soluble excipient. Two points of practical importance were involved. First, synthetic oestrogen tablets containing a suitable excipient can more easily be prepared on a large scale than tablets of pure oestrogen. Secondly, the technique would be greatly simplified if a successful treatment could be devised which necessitated only implantation, and not a second operation (often involving laborious exploration of the implantation site) for the removal of the tablets.

It was thought that tablets containing an appreciable proportion of a readily soluble excipient, such as lactose, might be absorbed quicker than pure oestrogen tablets of similar size, since the lactose would be completely dissolved within a short time of implantation, leaving sponge-like structures which might be more permeable to body fluids than compressed tablets containing no excipient. Even if the absolute absorption rates of comparable tablets of both types were the same, the tablets made up with excipient should disappear sooner than tablets of pure oestrogen, since the percentage absorption rate for the former would be the greater.

Experiments on rats [Parkes, 1942] had indicated that 50 mg. tablets consisting of 50 % hexoestrol, 49 % lactose and 1 % stearic acid (incorporated as a 'lubricant') would undergo complete absorption within 8-10 weeks. Tablets of this composition were therefore used in these experiments, since previous work quoted above had shown that in many cases in which positive lactation responses had been obtained, it was desirable that the operation of the oestrogenic stimulus should be limited to roughly this period. It was found, however, that under the conditions of these experiments, the tablets were absorbed much slower than anticipated, so that it became necessary to remove them. This made it possible, however, to obtain useful data on their rates of absorption in bovines.

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## EXPERIMENTAL

*Animals*

The main experiment was carried out on eight South Devon maiden heifers belonging to the Agricultural Research Council and maintained at Yelland Farm, Dartington Hall Ltd. These animals were purchased in the open market as 'bulling' heifers and nothing was known of their previous histories.

In addition, two dry cows at Old Parsonage Farm, Dartington Hall Ltd., and two belonging to the Agricultural Research Council's Field Station, Compton, were made available for experiment.

All animals were milked once daily, beginning 14 days after implantation. Twice-daily milking was started when the daily milk yield had reached 5 lb.

*Tablets and implantation technique*

The composition of the tablets used has been described above. Two sizes of tablet, averaging 50 and 250 mg. in weight, were used, and, save in one case (Pat 6), the effects of implants totalling 0.5 and 2.0 g. made up of tablets of each size were studied. After removal of the original implant, six of the heifers were subsequently treated with one of two types of implant consisting of 15 mg. tablets of pure hexoestrol.

Table 1. *Details of treatment and milk yields of heifers implanted in the first instance with tablets containing 50% hexoestrol*

Heifer	Original implant (50 % hexoestrol tablets) mg.	Subsequent implant (100 % hexoestrol tablets) mg.	Original implan- tation period days	Subsequent implan- tation period days	Duration of recorded lactation weeks	Maximum daily milk yield* lb.	Total recorded milk yield lb.
Daffodil	10 × 50	—	91	—	45	16	2702
Rose	10 × 50	25 × 15	56	84	—	(ca. 100 ml.)	—
Bluebell	2 × 250	100 × 15	56	84	44	10½	1556
Iris	2 × 250	100 × 15	84	70	24	2½	182
Snowdrop	40 × 50	—	91	—	45	21	3302
Tulip	40 × 50	25 × 15	56	77	44	10½	1449
Buttercup	8 × 250	25 × 15	56	43	45	7½	1295
Crocus	8 × 250	25 × 15	84	70	24	1½	121

\* Mean daily yield for 7-day period.

Table 2. *Details of treatment and milk yields of dry cows implanted with tablets containing 50% hexoestrol*

Cow	Breed	Implant (50 % hexoestrol tablets) mg.	Implan- tation period days	Duration of recorded lactation weeks	Maximum daily milk yield* lb.	Total recorded milk yield lb.
Dusky	Ayrshire × Friesian	2 × 250	70	11	¾	52
Rosabella	Ayrshire	2 × 250	118	25	3½	429
Pat 6	South Devon	6 × 250	84	9†	1½	49
Juliet 7	South Devon	40 × 50	112	71	25½	6928

\* Mean daily yield for 7-day period.

† Cow slaughtered 84 days after implantation, at which time her daily yield of secretion was slowly increasing.

All implantations were subcutaneous and the technique of insertion and removal of the tablets was similar to that described previously [Folley & Malpress, 1944a].

Details of the treatment given to the eight heifers are given in Table 1, and that accorded to the four cows in Table 2.

### *Determination of absorption data*

After removal, the tablets were cleaned, washed in distilled water, dried over  $\text{CaCl}_2$  and weighed after equilibration in air. Undamaged tablets and tablet debris were weighed separately. The whole implants, undamaged tablets and debris, were then quantitatively extracted with ether as described by Folley [1944] and the weights of the ghosts [Folley, 1942] determined by difference. By allowing for the weights of the ghosts, true absorption rates were thus determined. The supplementary implants consisting of 15 mg. tablets, received by six of the heifers, were not weighed after removal.

## RESULTS

### *Milk yield*

The lactation curves for heifers receiving in the first instance 0.5 g. implants are shown in Fig. 1 and those for heifers given 2.0 g. implants in Fig. 2. The points represent mean daily yields over successive 7-day periods and for each point the abscissa represents the last day of the period. Maximum and total yields are given in Table 1.

It will be seen that of the heifers receiving 0.5 g. implants of 50% hexoestrol tablets only one, Daffodil, gave a satisfactory response. Her yield rose rapidly to a peak of 16 lb. daily, the rise continuing after removal of the tablets but thereafter decreased rather quickly. Of the other three heifers in question, two gave slight responses which were increased somewhat by subsequent supplementary implants of 15 mg. tablets of pure hexoestrol, while the third, Rose, gave no more than 100 ml. of secretion even after a supplementary implantation. This heifer was, in the course of the experiment, diagnosed as suffering from staphylococcal mastitis.

Similarly, of the heifers treated with 2.0 g. implants of 50% hexoestrol tablets only one, Snowdrop, responded satisfactorily. In this case a peak yield of 21 lb. daily was reached, but once again the yield fell off rapidly giving an almost symmetrical lactation curve. Two others of these heifers were giving small amounts of secretion when the tablets were removed at 8 weeks, and in one case (Buttercup) the yield rose appreciably thereafter. This heifer did not respond well to a supplementary implantation but an appreciable response was given by Tulip.

The maximum and total yields of the four dry cows are given in Table 2. The two cows receiving 0.5 g. implants responded only slightly, as did the cow (Pat 6) which received  $6 \times 250$  mg. tablets, though her yield was increasing slowly at the time she was slaughtered. On the other hand, Juliet 7, which received a 2.0 g. implant, gave a satisfactory response. There was some delay before she came into milk, but at 8 weeks her yield rose rapidly to a peak of nearly 25 lb. daily. Removal of the implant was delayed until the yield was well past its peak and was falling rapidly, but immediately the implant was removed the yield rose again to an even higher peak. This animal milked well over a total period of about 60 weeks, during which

time she produced a total yield of approximately 693 gal. Eventually she became pregnant and consequently dried off.

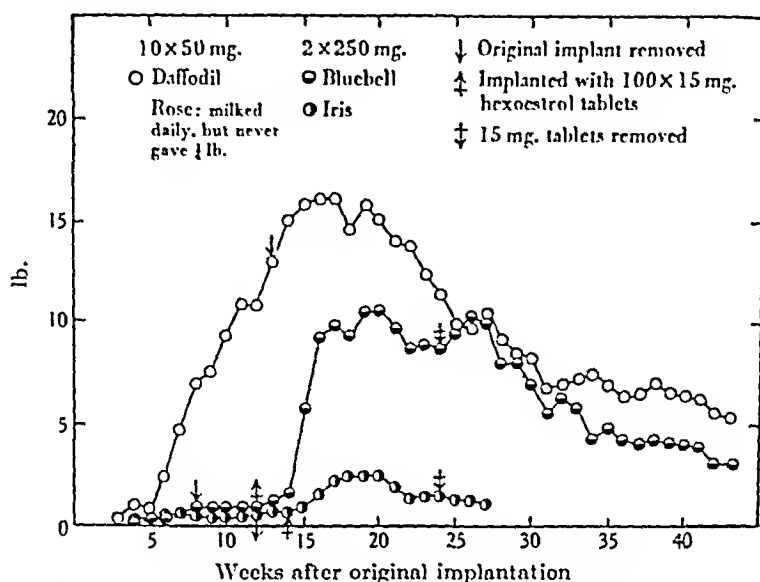


FIG. 1. Lactation curves of heifers implanted in the first instance with 0.5 g. implants consisting of tablets containing 50 % hexoestrol. (Mean daily yields for 7-day periods.)

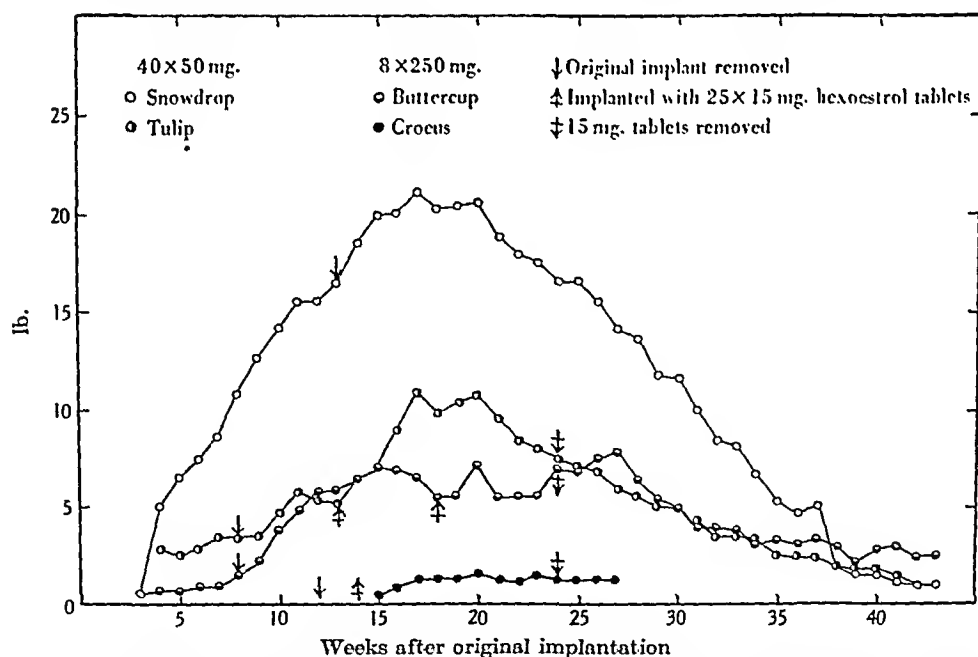


FIG. 2. Lactation curves of heifers implanted in the first instance with 2.0 g. implants consisting of tablets containing 50 % hexoestrol. (Mean daily yields for 7-day periods.)

#### Absorption data

The tablet absorption data for the heifers are given in Table 3 and for the cows in Table 4. Table 4 includes, in addition to data for the four cows referred to in Table 2,

Table 3. Absorption data for heifers implanted with tablets containing 50% hexoestrol

Implant (50% hexoestrol tablets)	Implant- tion period days	Wt. of implant mg.	Wt. of recovered implant mg.	Wt. of extract of implant mg.	No. of tablets recovered intact	Wt. of tablets recovered intact mg.	Esti- mated recovery of implant %	Wt. of ghosts in recovered implant mg.	Total absorp- tion mg.	Mean daily absorp- tion mg./day	Error in absorption determinations if wt. of ghosts is neglected %
Ueifer											
Daffodil	91	488	132	109	8	108	98	23	133	1.5	17
Rose	50	472	102	130	3	91	80	23	62	1.1	46
Bluebell	50	503	262	178	2	202	—	25	74	1.3	34
Iris	84	564	190	165	2	190	—	24	89	1.0	28
Snowdrop	91	1048	681	—	22	402	93	84*	332	3.7	27
Tulip	50	1964	785	696	37	767	96	90	258	4.6	36
Buttercup	50	1956	847	740	8	847	—	109	210	4.3	45
Crocus	84	1077	718	617	5	470	94	90	333	4.0	32

\* Owing to an accident during extraction the extract was lost. The ghost was in this case weighed direct.

Table 4. Absorption data for dry cows implanted with tablets containing 50% hexoestrol

Cow	Implant (50% hexoestrol tablets) mg.	Implan- tion period days	Wt. of implant mg.	Wt. of recovered implant mg.	Wt. of extract of implant mg.	No. of tablets recovered intact	Wt. of tablets recovered intact mg.	Esti- mated recovery of implant %	Wt. of ghosts in recovered implant mg.	Total absorp- tion mg.	Mean daily absorp- tion mg./day	Error in absorption determinations if wt. of ghosts is neglected %
Snowmalden	10 x 50	70	478	138	112	7	108	90	25	113	1.6	25
Dinky	2 x 250	70	508	264	171	1	104	98	33	86	1.1	42
Rosabella	2 x 250	118	483	123	166	2	123	—	17	136	1.2	13
Pat 6	6 x 250	84	1504	625	449	4	372	94	76	275	3.3	20
Juliet 7	40 x 50	112	1992	476	403	15	215	83	74	497	4.4	18

data for an additional cow at Compton which was implanted with  $2 \times 250$  mg. 50% hexoestrol tablets and subsequently injected with anterior pituitary extract.

It will be noted that there was a tendency for the tablets to become damaged *in situ*. This was particularly so in the case of the 50 mg. tablets, no implant of which was recovered intact; as regards the implants consisting of 250 mg. tablets, in four out of seven cases the correct number of undamaged tablets was recovered.

In cases where some tablets were fragmented *in situ* the total true absorption [see Folley, 1944] was estimated by the methods given below. In all absorption determinations the presence of 1% stearic acid in the tablets was left out of account.

Let  $w_1$  = original weight of implant,  $n_1$  = number of tablets implanted,  $n_2$  = number of tablets recovered intact,  $w_2$  = weight of the tablets recovered intact,  $w_3$  = total weight of the recovered implant,  $w_4$  = weight of the ether extract of the recovered implant, then

$$\text{Estimated final weight of implant had it remained intact} = \frac{w_2 n_1}{n_2} = w_5,$$

$$\text{Weight of ghosts present in recovered implant} = w_3 - w_4,$$

$$\text{Estimated weight of ghosts present in recovered implant had it remained intact} = \frac{w_5 (w_3 - w_4)}{w_3} = w_6,$$

$$\text{Total absorption} = \frac{w_1}{2} - w_5 + w_6.$$

$$\text{Estimated percentage recovery of implant} = \frac{100 w_3}{w_5}.$$

$$\text{For implants which were recovered intact the true total absorption was given by } \left( \frac{w_1}{2} - w_2 \right) + (w_2 - w_4).$$

The values so obtained and given in the various columns of Tables 3 and 4 are approximations which depend on the assumptions that the component members of a given implant were, at the outset, of uniform size and were all absorbed at the same rate. These assumptions are probably sufficiently near the truth to render valid the values obtained by the above methods in cases in which a good proportion of the tablets was recovered undamaged. This conclusion is borne out by the good agreement of the absorption rates for the four cases in Tables 3 and 4, in which the whole implant was recovered intact, with the estimated values for comparable implants which were damaged.

Even if the estimated absorption rates are near the truth it does not follow that the animals in which tablets underwent fragmentation *in situ* actually absorbed amounts of oestrogen closely approximating to the amounts calculated, since it would be expected that very small fragments would be absorbed quicker than undamaged tablets. However, the estimated values for the percentage recoveries of the implants, which were in most cases over 90%, indicate that this could not have happened to any great extent.

The absorption rates for the 0.5 g. implants range from 1.0 to 1.6 mg./day (mean 1.1 mg./day) and for the 2.0 g. implants the range is 3.7–4.6 mg./day (mean 4.2 mg./day). The agreement among individual values in each series is satisfactory considering the nature of the determinations. It is also noteworthy that the absorp-

tion rate of the 2.0 g. implants was approximately four times that of the 0.5 g. implants. Failure to allow for the weights of the ghosts would, in these determinations, have resulted in errors ranging from 13 to 46% (see Tables 3, 4).

*Effect of treatment on the reproductive organs*

The ovaries of the eight heifers and the cow Juliet 7 were examined by rectal palpation at regular and frequent intervals from the start of treatment until those animals which were retained for breeding became pregnant. All animals may be considered together, since there appeared to be no difference between those receiving 0.5 and 2.0 g. implants which could be ascribed to the differences in treatment.

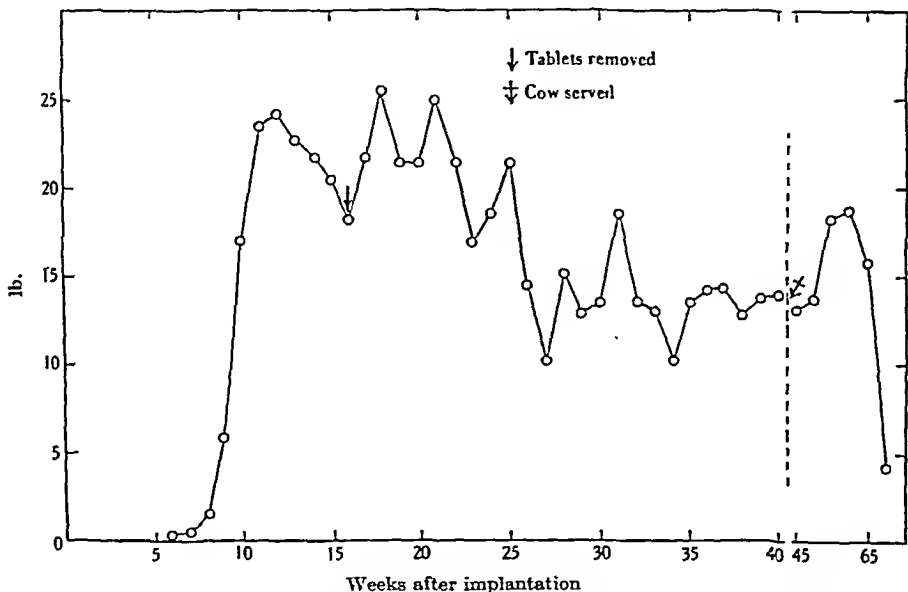


FIG. 3. Lactation curve of a dry cow, Juliet 7, implanted with 40 x 50 mg. tablets containing 50% hexoestrol. (Mean daily yields for 7-day periods.)

The ovarian changes were similar to those reported by Folley & Malpress [1944 *a, b*], in that there was in general a tendency for the ovaries to become quiescent shortly after implantation. In a majority of cases removal of the tablets was followed by the appearance in one or both ovaries of multiple large follicles or 'cysts'. During the implantation period most animals showed signs of oestrus at frequent and irregular intervals.

Soon after the removal of the second (supplementary) implants, three heifers, Rose, Iris and Crocus, which had never given more than slight amounts of milk, were sold; the remaining five were retained for breeding. Of these, four resumed normal, cyclic ovarian changes 4, 7, 10 and 16 weeks after cessation of oestrogen treatment and conceived at the first mating. All of these animals have since delivered bull calves, two being born dead. The fifth heifer, Snowdrop, had not resumed



a normal ovarian cycle 31 weeks after removal of the tablets despite frequent rupturing of the ovarian cysts per rectum; she was therefore discarded.

The cow, Juliet 7, exhibited ovarian cysts from 14 days after cessation of treatment. These were ruptured per rectum on four occasions, and she eventually resumed normal oestrous cycles 21 weeks after removal of the implant. This cow was mated and conceived at her third normal oestrus and eventually delivered a premature male calf (256 days).

Symptoms of nymphomania such as raised 'tail-head', relaxation of the pelvic ligaments, swelling of the vulva and vaginal discharge were exhibited to a varying degree by all animals. Though none of these animals was left in isolation, no pelvic fractures occurred. One heifer (Crocus) showed signs of vaginal prolapse when under the influence of the supplementary implant. This was most marked in the recumbent position. The condition disappeared without treatment.

#### DISCUSSION

In these, as in previous experiments on the artificial induction of lactation [Folley & Malpress, 1944*a, b*], an outstanding feature of the results was the wide individual variation in response. Only two of the eight heifers gave satisfactory responses to implants consisting of tablets containing 50% hexoestrol, one to a 0.5 g. implant and the other to a 2.0 g. implant. It is impossible to say whether or not the fact that in both cases the implant was composed of 50 mg. tablets was more than a coincidence. In each case the response was in sharp contrast to the responses of other heifers receiving identical treatment or comparable implants composed of 250 mg. tablets. Among the dry cows, the responses to 0.5 g. implants were slight, but the response of Juliet 7 to a 2.0 g. implant, again composed of 50 mg. tablets, was easily the best in this series of experiments. The cause of this variability in response is at present unknown, though it is suspected that the age of the test animal may be an important factor.

It will be seen from Figs. 1 and 2 that similar individual differences characterized the responses, to the supplementary implants consisting of 100 or  $25 \times 15$  mg. tablets of pure hexoestrol, of those heifers which were thus treated because they had responded poorly to the original implantation. Of the six animals given supplementary treatment only three showed unequivocal evidence of a resulting increase in yield. In two cases this increase was substantial, recalling similar findings by Walker & Stanley [1941].

As in previous experiments [Folley & Malpress, 1944*a*], evidence that in some cases an oestrogen implant tends to exert an inhibitory rather than a stimulative action after lactation has set in was provided by the present results. This was most clearly exhibited by the lactation curve of Juliet 7, which having reached its peak was declining sharply when the implant was removed. Withdrawal of the implant was immediately followed by an increase in milk yield to a higher peak than before. The lactation curve of Buttercup is also a good example of the phenomenon under discussion.

In view of the obvious desirability of reducing the oestrogen dosage to the lowest level consistent with a satisfactory response, in order that the risk of undesirable side-effects of treatment may be reduced to a minimum, it is interesting to note that

the three substantial responses were obtained with a considerably smaller expenditure of oestrogen than in any implantation experiments reported hitherto. Daffodil, Snowdrop and Juliet absorbed approximately 133, 332 and 497 mg. of hexoestrol respectively, thus comparing very favourably in this respect with animals giving comparable responses to tablets of pure oestrogen [Folley & Malpress, 1944a]. In the latter experiments, among animals which gave a total yield of at least 250 gal., the least amount of oestrogen absorbed was 1100 mg.

These experiments show that tablets containing 50% hexoestrol and 49% lactose are technically applicable to the purpose of induction of lactation in bovines, since they appear to show no greater tendency to break up *in situ* than small tablets of pure hexoestrol. Under the conditions of these experiments, however, they proved to be absorbed much slower than was anticipated from experiments on rats [Parkes, 1942]. The absorption rates of the four types of implant investigated, calculated per gramme of hexoestrol, were remarkably consistent considering the nature of such determinations and were of the order of 4 mg./day/g., from which it may be calculated that about 250 days would be necessary for the complete absorption of a 2.0 g. implant.

It is instructive to compare this value with the absorption rate of implants consisting of small tablets of pure hexoestrol. The only implants used by Folley & Malpress [1944a] which were comparable as regards the total surface areas of the component tablets were implants composed of 50 × 50 mg. hexoestrol tablets. Unfortunately, only three experiments are available for comparison, in two only of which the estimated recoveries were over 90%. The absorption rates in these two experiments were 9.2 and 8.0 mg./day, giving a mean value of 3.4 mg./day/g. These results were not corrected for ghost formation so that the true absorption values were probably a little greater. The average sizes of the two types of 50 mg. tablets under discussion were approximately the same, hence follows the unexpected result that the 50% hexoestrol tablets appear to be absorbed significantly slower than tablets of pure oestrogen of similar size despite the presumably more porous nature of the former after the lactose has been dissolved away.

As is to be expected from the sponge-like nature of 50% hexoestrol tablets after solution of the lactose, the tablets after removal were found to contain unusually dense ghosts. Thus the ghosts present in implants consisting of 2 × 250 mg. tablets, in the three cases in which the tablets were recovered intact, weighed 25, 24 and 17 mg. respectively (Tables 3, 4), which may be compared with a range of 4.5–7.5 mg. for ghosts present in single 250 mg. tablets of hexoestrol implanted into various laboratory animals [Folley, 1944]. In the case of tablets of the composition used in these experiments it is evident that failure to correct the absorption determinations for ghost formation would lead to considerable error.

#### SUMMARY

1. Experiments have been carried out on the use of subcutaneous implants of tablets containing 50% hexoestrol, 49% lactose and 1% stearic acid for artificial induction of lactation in maiden heifers and dry cows. Individual variations in response were large, but satisfactory responses were given by two heifers and one dry cow to low total doses of oestrogen.

2. The responses of six heifers giving small amounts of secretion to further implants consisting of pure hexoestrol tablets were also variable. In two cases marked increases in yield resulted.

3. Symptoms of nymphomania were observed in all animals but none suffered pelvic fracture.

4. Of six animals retained for breeding after cessation of treatment, five became pregnant to the first mating. All delivered male calves, two being born dead and another prematurely.

5. In the case of implants consisting of 50 mg. tablets containing 50 % hexoestrol, there was a tendency for some of the tablets to become broken *in situ*, though not to any greater extent than small tablets of pure hexoestrol.

6. Under the conditions of these experiments tablets containing 50 % hexoestrol were absorbed more slowly than pure hexoestrol tablets of comparable size.

We are much indebted to Dr W. K. Slater for enabling the major portion of this work to be carried out from The Laboratory, Dartington Hall Ltd., and for valuable co-operation in numerous ways and also to the Agricultural Research Council and the Medical Research Council for grants covering the expenses of the investigation. We are also grateful to Dr A. S. Parkes for putting his results with 50 % hexoestrol tablets in rats at our disposal. Our best thanks are due to Mr H. T. Fawns and Mr J. B. E. Paterson for much valuable assistance and to Mr H. Tope for the care of the heifers. Permission to use the animals at Compton was kindly given by the late Mr W. G. Dunkin, and for their care we are indebted to Mr Maltby. Miss D. Woolcott rendered valuable technical assistance.

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# OESTROGEN TREATMENT OF CATTLE: INDUCED LACTATION AND OTHER EFFECTS

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(Received 17 December 1943)

## INTRODUCTION

### *Normal onset of lactation*

In the majority of species investigated it would appear that oestrogen stimulates mammary duct growth, but that alveolar development requires luteal secretion. To this the cow and the goat are evidently exceptions, and perhaps this is not unconnected with the fact that they have been domesticated for milk production. A marked difference between the cow and the goat, which is perhaps rather singular, concerns the development of the mammary gland.

Among goats of high milking strains, though not with the poorer class of animal, it is the rule rather than the exception that they may develop an udder and come into milk without ever having been mated. The onset of such a lactation comes commonly in the spring, which is also the end of the breeding season, and the yield, though less than what would be given after kidding, may yet be as great as that produced by a poorer type of animal in a normal lactation.

Cases of similar lactation in cattle have been recorded, but they are by no means common. We have heard of two such animals recently: one of these, a Jersey heifer, was about 4 months in calf at the time, the other was a Guernsey which had been bought as an in-calf heifer and was then showing some udder development; it was found to be not pregnant, and the history suggests very strongly that it had cystic ovarian follicles. About a year after purchase it suddenly came into milk and yielded up to 15 lb. daily; it was seen when killed some months later and was then in calf from a service subsequent to the onset of lactation. Except that there were two corpora lutea of pregnancy and only a single foetus the reproductive tract was then entirely normal.

The udder of a virgin heifer is mostly a pad of fat; with successive oestrous cycles there is some development, principally duct extension and arborization which is not greatly accelerated in the first months of pregnancy; in the fifth month the rate of development greatly increases and thickening of the alveoli occurs [Hammond, 1923; Turner, 1931]. A little secretion can usually be obtained from the virgin gland; this is a thin serous fluid containing the characteristic milk substances caseinogen and lactose, and with a relatively high globulin content; the nature of this secretion does not change until about the 20th week of pregnancy, when it becomes very thick and honey-like in appearance, the globulin becoming very high and caseinogen and lactose much reduced; if this is drawn off, the secretions again become colostrum in type, but if not it remains thick until the end of pregnancy, when it is diluted with milk to form colostrum [Woodman & Hammond, 1922, 1923].

Asdell [1925] milked a heifer throughout her first pregnancy; he observed the thick secretion slightly earlier and it did not appear simultaneously in all four quarters. Thereafter the yield rose steadily, slowly at first, quickly in the last few weeks, and the globulin content correspondingly fell; he also noted the thick secretion to occur in a goat at the 11th week. Turner [1931] similarly followed several heifers; one came into milk quite rapidly and was giving 10 lb. a day 120 days before calving, the others increased slowly and steadily, only rising rapidly in the last 3 weeks; the yield from a cow remained low right up to parturition; he also noted the volume of secretion to be unevenly distributed between the quarters while the yield remained low.

Turner, Frank, Lomas & Nibler [1930] followed the course of urinary oestrogen excretion in pregnant cows; the amount rose slowly at first and then with increasing rapidity. In the latter months of pregnancy the daily output was higher in dairy than beef animals; so, however, was the volume of urine. It is at about the 5th month, when the histological and secretory changes are observed in the udder, that the excretion curve of Turner *et al.* takes a sharp upward trend; at this time also the yields of pregnant cows fall off rapidly [Gavin, 1913; Hammond & Sanders, 1923], there having been a slight decline following fertile service.

Drummond-Robinson & Asdell [1926] removed the corpora lutea from goats in their first pregnancy and observed that the abortion was accompanied by the onset of lactation if the high-globulin stage of mammary development had been reached, but not otherwise. This applies also in the case of aborting heifers.

### *Experimental induction of lactation*

Evans [1932] gave a series of pituitary-extract injections and obtained considerable yields from three virgin and one dry goats, the yield apparently being maintained after the cessation of treatment; a heifer similarly treated is stated to have given 15 lb. a day. Catchpole, Lyons & Regan [1933] tried to repeat this result with fairly young heifers; they obtained only small amounts of milk and the yield fell off after cessation of the injections. Least response was obtained with the youngest animals.

Induction of lactation with oestrogen has been a more recent development. In the goat de Fremery [1938] obtained lactation with pituitary extract following oestrogen preparation; with oestrogen alone considerable amounts of secretion have been obtained with udder inunction [Folley, Scott-Watson & Bottomley, 1940, 1941*b*] and with daily injections [Lewis & Turner, 1940]. The latter authors observed also depression of yield and drying off with treatments of lactating animals: later [1942] they obtained, using a tablet implant, a second lactation from an animal treated when a kid.

In cattle an enrichment effect separable from transient depression of yield with higher doses has been reported [Folley, 1936; Folley *et al.* 1941*a*] as an effect of oestrogen on established lactation. A small amount of secretion was obtained from a virgin heifer by udder inunction [Folley *et al.* 1940]. With a combination of injection and inunction Lewis & Turner [1942] obtained just over a litre a day from a dry cow having only one normal quarter.

Two heifers, one of which also received testosterone, treated by Walker & Stanley [1941] after a prolonged course of stilboestrol injections reached levels of 14 and

16 lb. a day; another heifer [1940], previously treated with pituitary extract without apparent mammary effect, was brought into milk by ten 5 mg. daily injections of stilboestrol dipropionate.

Rowson [personal communication] has observed copious secretion following stilboestrol treatment of a heifer with pyometra, and has succeeded in bringing normal heifers into milk with a short course of injections. There have been several reports of induced lactation following use of stilboestrol in veterinary practice.

### *Present experiments*

A heifer supposedly having a retained mummified foetus was treated with oestrogen and came into milk; however, similar treatment to two other animals was ineffective. In the course of some experiments with rabbits [Hammond, Jr., unpublished] the condition of the uterus after excessive oestrogen dosage suggested that commonly found in cows having cystic ovaries. A group of heifers treated with stilboestrol tablet implants did not show the anticipated effect on the uterus, but there was very marked udder development and there was milk in the teats.

Treatment in this way of cows in declining lactation led to prompt drying off; two heifers which had failed to get in calf were brought into milk, gave yields approaching 2 gal., and were got in calf; a number of other heifers also gave good yields. It was through the generosity of the Earl of Iveagh, C.B., that the majority of these animals were made available for experiment, and grateful acknowledgement is made of this fact. At this point the investigation received the direct support of the Agricultural Research Council and a trial planned involving forty-eight heifers, together with a like number under the supervision of Dr Folley of the N.I.R.D., Reading. Other work has been concerned with treatment of those heifers not giving satisfactory yields with tablet implants alone, alternative methods of tablet implantation, and continued experiments with cows.

## MATERIAL AND METHODS

### *Animals used*

The animals used are listed in Table 1: all were privately owned and kept on farms in the eastern counties of England. With the exception of nos. 37 and 38 which had been dried off early after a poor first lactation, the cows were for the most part rather good animals which had failed to get in calf. The heifers also were those which had not become pregnant, usually after running with the bull during the winter months; the extent to which they had been so selected for infertility is illustrated by the frequency among them of 'white heifer disease' and analogous conditions; not all such animals encountered have been treated. As regards age, breed and quality they were rather mixed; most of the heifers were from 24 to 36 months old, but a few fall outside this range, above and below. They were in milk-recorded herds and, from this fact, and because some would have been, or had been, thought worth keeping on for another season, can be considered rather above the average level in dairy quality.

The breeds of the various animals were as follows: Jersey, N, 100, 112-115, 121, 126-128, 132, 134-136; Aberdeen Angus cross, 1-5; Guernsey, 11, 13-16, 21-24, 65-70, 84; Red Poll, 25, 26, 88, 89, 118-120, 124; Ayrshire, 29, 30, 83, 85-87, 105.

Table 1. *Induced lactations*[illegible]

	Freemartin?													Milk only in hind quarters of milker	
51	"	1-07 S	74	14-1	—	1-1	2-3	—	08	—	13	3-5	142	—	
52	"	0-88 S	74	11-6	—	1-1	1-4	—	47	—	13	3-1	100	—	
53	Uterine adhesions	0-81 S	67	8-8	—	4-3	5-8	7-4	160	—	21	5-6	682	—	
54	Normal heifer	1-12 S	67	11-5	—	5-0	7-0	10-7	213	—	21	10-0	1015	—	
55	"	0-85 S	67	8-8	—	0-2	4-3	14-5	51	—	21	12-1	1035	Not pregnant	
56	"	1-00 S	67	10-0	—	4-5	11-1	12-8	229	—	21	12-0	1214	Not pregnant	
57	"	1-11 S	74	12-0	—	—	—	—	0	—	14	0	—	—	
58	As white heifer	1-23 S	67	12-0	—	5-1	11-7	13-0	368	—	21	10-0	1108	Not pregnant	
59	White heifer	0-71 H	68	12-3	—	17-3	22-8	21-1	703	2508	01	Dry	6018	—	
60	Normal heifer	0-07 H	101	0-6	—	7-1	13-4	14-5	336	—	27	10-8	1038	—	
61	"	1-31 H	102	12-8	12-3	17-0	17-1	16-0	870	1060	33	10-1	3020	—	
62	"	0-05 H	67	14-2	0-4	15-7	15-5	16-8	720	2014	53	Dry	4217	In calf	
63	"	0-00 S	102	0-5	2-0	2-3	2-0	17-1	110	1003	50	8-7	3510	Aborted	
64	"	0-07 H	67	10-0	—	15-0	15-0	15-7	600	1770	57	Dry	4142	Calved	
65	"	2-11 H	102	23-8	—	—	—	—	0	1277	50	7-6	4217	In calf	
66	"	0-70 S	67	11-3	14-0	21-5	13-5	20-7	790	2611	—	1-1	12-6	1207	—
67	"	1-51 H	102	15-1	—	10-7	23-4	20-7	790	2611	—	1-1	12-6	1207	—
68	"	0-86 H	68	12-6	1-6	7-0	8-7	8-6	301	—	25	4-7	1130	In calf	
69	"	1-18 H	68	17-3	1-0	11-3	12-0	11-2	460	—	25	0-8	1535	In calf	
70	"	1-01 S	101	10-0	3-0	10-6	6-1	5-0	416	—	20	3-0	781	—	
71	"	1-01 S	68	14-8	—	—	—	—	0	—	25	0	0	—	
72	"	1-10 H	101	11-5	2-0	0-6	15-0	15-1	402	1065	34	12-8	2773	—	
73	"	1-10 H	101	11-5	2-0	0-6	15-0	15-1	402	1065	34	12-8	2773	—	
74	"	0-58 S	61	9-1	0-3	0-5	7-6	8-0	130	018	34	2-0	1161	—	
75	Aborted at about 5 months	0-58 S	61	9-1	0-3	0-5	7-6	8-0	130	018	34	2-0	1161	Most of milk from one quarter	
76	Normal heifer	0-55 H	61	8-0	0-3	11-2	16-5	15-6	408	1680	31	11-3	2806	Aborted	
77	"	1-24 H	105	12-0	6-0	0-0	10-1	6-4	451	1000	37	3-3	1615	Aborted at 4 months	
78	"	0-06 S	105	6-2	18-2	21-0	27-0	22-1	1270	2760	60	7-8	6850	Cystic ovaries after tablet removal	
79	"	2-17 H	105	20-7	4-0	11-5	16-5	11-1	573	3128	58	Dry	4281	Shipped calf about 3 months after first service	
80	"	0-38 H	60	9-7	6-6	21-7	27-8	25-3	1029	3318	44	Dry	5241	—	
81	"	0-72 S	60	12-0	7-0	21-7	29-7	26-0	1007	3660	46	Dry	6195	—	
82	"	1-31 H	60	13-3	11-3	17-0	23-2	16-7	612	2706	41	8-0	4567	Cystic ovaries after tablet removal	
83	"	1-35 H	60	20-8	2-7	18-6	20-3	17-7	690	2421	33	10-8	3363	One quarter blind	
84	"	0-72 H	60	7-2	—	17-2	20-3	19-4	723	2228	54	Dry	4355	—	
85	"	S*	60	?	—	15-3	10-1	21-1	610	2746	61	Dry	6089	—	
86	"	1-10 S	60	12-1	—	—	—	—	0	Not recorded, estimated	Peak 25 lb.	10-7	6808	Came into milk a month after tablet removal	
87	"	0-77 H	65	11-8	0-1	23-5	26-6	26-8	1080	4065	58	10-7	6808	Developed cystic follicles after several cycles	
88	"	0-52 H	65	7-9	c. 1-0	21-6	28-1	28-6	608	3536	58	14-1	6223	Not pregnant	
89	"	1-10 H	66	14-4	c. 0-2	c. 0-5	—	—	c. 10	—	12	2-0	c. 30	In calf	
90	"	0-88 S	57	15-1	1-1	6-4	7-5	—	300	—	14	11-0	568	? Persistent corpus luteum	
91	"	S*	35?	?	1-3	1-5	—	—	—	—	0	1-8	60	—	
92	"	0-67 S	62	10-7	13-0	22-1	22-1	21-3	1016	2109	34	14-5	3875	Calved, twins	
93	"	0-03 H	60	9-1	9-0	16-0	14-8	12-5	709	1690	31	11-1	3716	Calved	
94	"	1-39 H	62	22-4	0-0	4-3	5-1	6-3	108	1307	31	12-6	1047	Not pregnant	
95	"	1-13 H	69	14-5	0-9	2-0	3-4	5-3	169	1029	31	8-3	1376	Pregnant	
96	"	0-00 S	128	c. 7-0	0-1	13-6	13-5	—	692	—	13	16-2	1036	Tablets in kidney fat	
97	"	0-22 S	153	c. 5-0	c. 0-2	c. 0-2	c. 0-2	c. 0-5	—	—	23	c. 0-5	—	Killed at 152 days. Tablets in flank fat	
98	Heifer with cysts 85 days after first implant	0-20 S	52	c. 4-0	c. 0-2	c. 0-2	c. 0-2	c. 0-5	—	—	—	—	—	—	



Table 1 (cont.)

No.	Condition before treatment	Total dose (g.) and substance	Duration of implant days	Mean daily dose mg.	Mean daily yield in week after implantation (lb.)							Total yield in weeks (lb.)		No. of services	If in calf	Remarks		
					Mean daily yield in week after implantation (lb.)							Total yield in weeks (lb.)						
					4	7	10	15	1-10 11-30			Daily yield (lb.)						
					4	7	10	15	1-10 11-30			No.	(lb.)	Total (lb.)				
121	Heifer with cysts	0.91 S	84	10.8	2.0	6.9	9.1	11.1	313	—			16	10.8	775	—	—	Killed at 151 days. Tablets in flank fat
124	"	0.50 S	154	c. 3.3	—	c. 0.2	c. 0.2	c. 0.5	—	—			22	c. 0.2	—	—	—	—
125	Normal heifer	1.46 S	131	11.1	—	—	—	—	—	—			33	0	—	—	—	—
128	Heifer with cysts	0.72 S	54	13.3	—	—	—	—	—	—			16	0	—	—	—	Yield at 20 weeks c. 22 lb./day
129	Normal heifer	1.35 H	110	12.3	—	—	—	—	—	Not recorded			—	—	—	—	—	—
130	"	1.01 H	110	9.2	4.6	12.7	15.8	15.6	585	2310	41	16.0	4214	—	—	—	—	
131	"	4.6 H	45?	?	8.6	15.0	17.5	23.0	722	2214	40	13.3	4473	3	In calf	Pregnancy not checked	—	
132	"	1.67 H	103	15.3	3.0	6.9	9.0	11.2	323	2427	42	17.4	4312	2	Not pregnant	—	—	
133	"	1.40 H	110	12.7	—	c. 2.0	c. 4.0	c. 4.0	c. 200	c. 900	41	22.7	c. 2120	—	—	—	—	
134	"	1.58 H	110	14.4	—	—	c. 0.2	c. 0.2	—	c. 800	41	17.0	c. 1910	—	—	—	—	
135	"	1.35 H	110	12.3	—	c. 0.2	c. 0.3	c. 0.4	c. 10	c. 400	41	11.5	c. 1200	—	—	—	—	
136	"	2.00 H	110	18.2	—	c. 0.2	c. 0.2	c. 0.2	c. 5	c. 200	41	21.0	c. 2110	—	—	—	—	
141	"	1.43 S	62	23.1	3.2	5.6	6.4	—	274	—	10	6.4	271	—	—	—	—	
I. (b) Heifers not milked at signs of secretion first appearing																		
Not milked at all																		
1	Normal heifer	0.59 P	52	8.0 S	—							—		—	—	Killed at 52 days		
2	"	0.10 S	52	3.7	—							—		—	—	Killed at 52 days, persistent c.L.		
3	"	1.39 S	45?	?	—							—		—	—	Killed at 52 days		
4	"	0.42 S	52	8.1	—							—		—	—	Killed at 52 days, control		
5	"	—	—	0	—							—		—	—	Killed at 52 days, control		
27	"	0.42 S	40	10.5	—							—		—	—	Killed at 40 days		
28	"	0.22 S	40	5.6	—							—		—	—	Killed at 40 days		
137	"	1.74 S	105	10.6	—	—	—	2.5	—	1701	39	13.1	2879	1	Pregnant	Milking started in 12th week. Yield rose as 129		
138	"	0.68 S	58	11.7	—	—	—	1.4	—	1519	20	13.9	1519	—	—	Died—lung haemorrhage		
139	"	1.68 S	105	16.0	—	—	—	—	—	—	—	—	—	—	Yield rose 3 weeks after tablet removal	—		
I. (c) Cows and previously treated heifers																		
6	Giving 17 lb./day	0.29 P	34	7.5 S	c. 0.4	—	—	—	—	—	—	—	—	—	Killed at 34 days, went dry in 8 days			
7	Giving 15 lb./day	0.34 P	34	8.9 S	c. 0.4	—	—	—	—	—	—	—	—	—	Killed at 34 days, went dry in 6 days			
12	Dry cow	0.21 S	69	3.1	—	—	—	—	0	—	13	0	0	—	Killed at 93 days			
13	End of lactation	0.04 S	63	0.6	No marked effect on yield							—		—	—	—		
14	"	0.13 P	63	1.5 S	"							—		—	—	—		
15	Dry cow	0.92 P	63	10.3 S	—	—	—	—	0	—	12	0	0	—	Killed at 87 days			
16	Giving 14 lb./day	0.41 S	63	6.5	—	—	—	—	0	—	12	0	0	—	Killed at 87 days, dry in 19 days			
33	Dry cow (cysts)	2.11 S	102	20.7	—	—	—	—	0	—	20	0	0	—	Yield rose following injection on day 107			
37	Young dry cow	1.30 S	87	14.9	0.7	2.3	7.8	7.1	177	2557	33	19.0	3147	2	Calved	—		
38	"	1.84 S	87	21.2	2.1	21.0	20.1	18.6	752	—	17	17.5	1559	—	—	—		
39	Dry cow	3.48 S	87	40.0	—	19.1	22.6	23.8	610	2710	32	13.5	3011	—	—	—		
40	Giving 10 lb./day	2.10 S	90	22.1	—	—	—	—	0	1563	32	6.1	1619	2	Not pregnant	Dry in 7 days		
41	Giving 9 lb./day	3.12 S	90	31.5	—	1.5	8.9	11.3	151	1788	32	7.0	2010	1	Not pregnant	Dry in 8 days		
42	Giving 14 lb./day	1.20 S	90	13.1	—	—	—	—	0	—	10	0	0	—	Dry in 9 days			
61	Dry cow (cysts)	1.55 S	104	14.9	2.0	4.5	5.1	12.6	203	—	29	8.6	1613	1	Not pregnant	—		
105	Heifer 85 days after implant	0.54 S	54	10.0	—	c. 0.5	—	—	—	—	8	c. 0.5	—	—	See 2 (a)	—		
106	Heifer 85 days after implant	0.30 S	54	5.6	—	—	—	—	—	—	8	0	0	—	See 2 (a)	—		
114	Dry cow	0.12 S	7	17.1	Died—choked by cleanings							—		—	—	Expelled mummified foetus day 7		
126	"	0.69 S	43	16.1	—	—	—	—	—	—	7	0	0	—	—	—		
127	"	0.88 S	53	16.6	—	—	—	—	—	—	8	0	0	—	—	—		

No.	Condition before treatment	Total dose (g.) and substance	Day after implant palpated when ovaries		Mean daily yield in week after implantation (lb.)							Total yield in weeks (lb.)		No. of services	If in calf	Remarks	
			Last inactive	First active	1					2. (a) 'Intrapartition' tablet implants. Heifers		1-10	11-30				
					4	7	10	15	1-10	11-30	No.		(b.)				
61	As 'white heifer'	50 x 0.05 S	—	31	c. 1.5	31.5	29.3	28.1	138.1	380.1	—	42	25.3	—	—	Killed at 31 days	
101	Normal heifer	3 x 0.11 S	290	—	18.7	31.5	29.3	28.1	138.1	380.1	—	46	18.7	5482	—	—	
102	"	2 x 0.11 S	—	62	—	8.5	22.7	20.1	488	2752	—	43	16.3	6111	4	In calf	
103	"	3 x 0.11 S	82	137	—	21.5	30.1	28.0	758	3692	—	26	0	0	—	Not pregnant	
104	"	2 x 0.11 S	62	81	—	—	—	—	0	—	—	12	0	0	—	—	
105	Heifer with cysts	3 x 0.11 S	—	62	—	—	—	—	0	—	—	26	12.3	1913	—	See 1 (c)	
106	Normal heifer	2 x 0.11 S	162	62	1.2	7.5	11.5	14.5	335	—	—	55	18.2	5180	2	See 1 (c)	
107	"	2 x 0.11 S	—	62	—	—	c. 10.0	0	c. 1000	—	—	51	19.0	3360	—	After two injections, second on day 83, came into milk	
108	"	2 x 0.11 S	—	—	—	—	—	—	0	c. 100	—	10	0	—	—	Killed at 101 days	
109	"	3 x 0.11 S	273	—	—	—	—	—	0	—	—	10	c. 1.0	—	—	—	
110	"	2 x 0.11 S	—	62	c. 1.0	—	—	—	0	—	—	15	1.1	85	—	Calved at term and in calf again	
111	"	2 x 0.11 S	—	62	—	—	—	—	85	—	—	—	—	0	—	—	
112	"	2 x 0.11 S	117	229	1.8	1.2	1.1	1.1	—	—	—	—	—	—	—	—	
117	Heifer 2 months in calf	3 x 0.11 S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
118	Normal heifer	2 x 0.31 S	27	32	—	15.0	17.8	17.3	591	2225	—	40	Dry	3193	3	Not pregnant	
119	"	2 x 0.31 S	102	115	—	3.2	11.3	11.5	231	1878	—	40	Dry	3011	3	Persistent c.l. removed on day 32	
120	"	2 x 0.31 S	16	63	2.0	9.5	17.0	10.1	401	2913	—	31	20.5	5311	0	Not pregnant	
122	"	3 x 0.31 S	232	—	—	—	—	3.1	0	—	—	18	5.7	150	—	Yield later rose to estimated 20 lb./day	
123	White heifer	3 x 0.31 S	92	—	—	—	—	—	0	0	—	32	0	0	—	—	
2. (b) 'Intrapartition' implants. Cows																	
84	Dry cow	110 x 0.015 S	370	408	3.5	11.9	11.1	14.0	408	1028	—	63	18.7	6597	—	—	
113	"	29 x 0.05 S	67	117	5.2	7.1	9.6	—	388	—	—	13	11.7	605	—	—	
115	"	30 x 0.05 S	—	53	2.1	—	—	—	—	—	—	4	2.1	22	—	Mummified calf expelled within 53 days	
3. Animals injected but not implanted																	
Injections																	
S	Heifer overbue to calve	1st	2nd	3rd	12.0	17.1	—	—	—	—	—	7	17.1	481	—	Thick secretion in teats when first injected	
		2.5 T	0.07 S	0.01 S	—	—	—	—	—	—	—	—	—	—	—	—	
		0.5 S	0.1 S	0.1 S	—	—	—	1.9	0	171	33	3.8	559	1	Not pregnant	—	
62	Dry cow	0.5 S	0.1 S, P, B	0.1 S, P, B	—	—	—	0.1	12.0	51	1101	33	4.5	1551	1	Secretion started day after third injection	

Abbreviations used: \* extrusion of tablets; S, stillhoestrol; P, stillhoestrol di-n-butylate; B, stillhoestrol di-n-butylate; c, about; c.l., corpus luteum.

106, 117, 125, 131; Friesian, 33, 40, 41, 44, 71, 91, 92, 96-99, 101-104, 129, 130, 133; the remaining sixty-nine animals were Shorthorns or Shorthorn crosses, except 105, Guernsey  $\times$  Ayrshire, and 108, Jersey  $\times$  Friesian.

The management of the animals has, of course, varied slightly from farm to farm. The giving of extra food, as with heifers shortly before calving, has been recommended for the first few weeks of treatment; in general this has been done. With certain exceptions, to be mentioned later, instructions were given for milking to be started as soon as the udder filled out and became tense. During the period of treatment some have been run with the rest of the herd, some as a separate small group, and some kept in stalls or boxes. In Table 1 the numbers of those animals are bracketed together which were under the same management and are also comparable as regards age or breed, or both.

### *Technique*

Tablets for implantation were shaken free of dust, counted out and the total weighed, and, if there were any number to be implanted, put in columns into glass tubes with internal diameter just greater than that of the tablets, the tubes being plugged with cotton-wool and labelled. The site for subcutaneous implants in most of the first thirty animals was at the side of the neck, but for the later implants a site just behind the shoulder was always chosen. Implantation and removal were done under local (Procaine) anaesthesia.

An incision 2-3 in. long, running downwards and forwards, was made in the shaven patch of skin. The underlying fascia were then picked up with forceps, a small hole made therein, and this extended downwards and backwards, with the finger or suitable blunt instrument, into a pocket large enough to hold the tablets, and not too near to the skin incision.

The plug being removed from one end of the tube, this was inserted into the pocket and the tablets run out; the pocket was then closed by a running suture of fine silk and the skin wound by three or four separate strong silk sutures. Healing was nearly always by first intention; however, in four cases, probably owing to stray hairs, there was some discharge, with extrusion of some or all of the tablets. The grouped tablets became surrounded by a single tough fibrous capsule; occasionally there was a small capsule containing a few tablets an inch or so away from the main body; it was usual also for there to be one or two separately encapsulated tablets near the suture in the fascia. In three cases (nos. 17-19) tablets became widely scattered.

Implants into the peritoneal cavity were made with trocar and cannula through the right flank, sufficiently low to avoid the kidney fat—not always easy in fat animals. Sucking noises on removal of the trocar, due to respiratory movements, usually indicated that the cannula extended into the body cavity; this could be confirmed by inspection. Tablets would not run straight through the cannula, but had to be pushed through with a rubber-tipped plunger.

In removing subcutaneously implanted tablets the skin incision was made directly over the capsule enclosing the tablets, care being taken not to cut into it. The capsule was dissected out whole, and the surrounding area carefully explored with the finger tip for outlying tablets—the smallest used being not always palpable through the skin.

The tablets after being dissected out were cleaned by shaking in one or two changes

of water, any matter still adhering being wiped off with moist cotton-wool. Not quite all foreign matter is removed from the tablet in this way, but the amount left is small and probably about counterbalanced by losses in washing. The clean tablets were then spread on a dish, left to dry for a week at room temperature, and then weighed, whole and chipped tablets separately. It was rather unusual with 15 and 25 mg. tablets for there to be 100% recovery of whole tablets; apart from tablets being broken at implantation and removal, and probably also *in situ*, there was sometimes a small number of tablets unaccounted for, and which must have been completely absorbed.

The animals were all examined per rectum at the time of implanting and the condition of the ovaries noted; all have also been so examined during the course of and after the end of treatment, but the frequency and intervals at which this has been done have varied with different animals.

Milk samples were taken at weekly intervals from a small number of animals and the development of secretion followed; some of the heifers were also included in routine herd sampling.

#### *Substances used*

Tablet implants have been made with stilboestrol,\* hexoestrol and, in five cases, stilboestrol dipropionate; the tablets contained no excipient except in the case of stilboestrol tablets weighing approximately 1 g. and 340 or 410 mg. to which 1% of stearic acid had been added; details of tablet sizes and shapes will be given later. Substances injected in arachis oil were stilboestrol 20 mg./ml., stilboestrol dipropionate 10 mg./ml., stilboestrol di-*n*-butyrate 20 mg./ml.; triphenylchloroethylene given to one animal was in saturated solution in sesame oil. The preparations of pregnant mare serum, human pregnancy urine and posterior-pituitary extracts used were all easily water soluble; the chorionic gonadotrophin was given intravenously; all other injections were subcutaneous.

### RESULTS

#### *Outline of experiments*

(a) Nos. 1-5 were a uniform group of beef heifers, about 34 months old, one of which was left as a control and the others implanted with stilboestrol or the dipropionate. They were killed after 52 days.

(b) Nos. 6 and 7, two cows in milk implanted with stilboestrol dipropionate, speedily dried off and were killed after 34 days; during the last week they were milked and yielded a small amount of colostral secretion.

(c) Nos. 8-11, four barren heifers, were implanted, two with a small number and two with a fairly large amount of stilboestrol tablets. Milking was started in all at 24 days, and tablets removed at 55 days. The two with lower dosage, one of which gave a small amount of milk and the other only a little serous secretion, were killed at 94 days. The other two were got in calf and have since calved twice each.

(d) Nos. 12-16, five cows, were implanted with different amounts to give a daily dose range of 0.6-10.3 mg. of stilboestrol. Those with the highest and median doses were dry; the others were at the end of lactation. After 63 or 69 days tablets were removed and they were killed 25 or more days later. That in milk given the highest

\* Throughout the paper this term refers to the diethyl compound: 4:4'-dihydroxy- $\alpha$ : $\beta$ -diethylstilbene.

dose was dried off; the yields of the other two did not show an effect not attributable to seasonal dietary changes.

(e) Nos. 17-20, four heifers, each implanted with the same amount of stilboestrol, gave a poor secretory response; no. 18 was the best and reached 15 lb./day. Following injection, no. 20 rose to 2 gal. and had the tablets then removed. The three others had the tablets left in and these had been completely absorbed when the three were killed after some 300 days. No. 17 received additional injection treatment and no. 19 a further implant. Milking was commenced before any sign of udder distension appeared. In this and the two succeeding groups observations were made weekly for some months and then at monthly intervals.

(f) Nos. 21-24, four heifers, were implanted, nos. 21 and 23 with 50, 22 and 24 with 150, stilboestrol tablets; nos. 22 and 23 were giving rather better yields than the other two when their tablets were removed at 79 days, in nos. 21 and 24 the tablets were left for 113 days.

(g) Nos. 25 and 26, two heifers, first implanted with 100 and 50 tablets respectively, were after 20 days, at which time the former had started to milk, given a further implant of 50 and 150 tablets.

(h) For intraperitoneal tablet implants nos. 27, 28 and 64 gave preliminary information on tablet absorption and technique, and nos. 84, 100, 113-115, with 140 (a bull), added further data. The main experiment was with heifers 101-112 and 116-124, except 121, and in these an attempt was made, sometimes—mainly through movement of the animal at a critical moment—unsuccessfully, to insert either two or three tablets into the abdominal cavity. The tablets weighed 410 or 340 mg. each, and the absorption time was to have been determined by periodic examination of ovarian activity; absorption was unexpectedly high, and in the first group a good estimate was not obtained of the time for absorption.

(i) The 'planned experiment', in which heifers 31, 35, 36, 43-49, 53-55, 59, 60, 65-71, 73-83 and 85-99 were used, was largely based on unrealized hopes of complete tablet absorption within the required time. 15 and 25 mg. tablets were used, equal numbers treated with stilboestrol and hexoestrol, and in each group the tablets removed from half at 60 and the other half at 100 days. This plan was subsequently modified to the extent that nos. 43, 53 and 55, which were not responding well, were given five to seven weekly injections of 100 mg. of stilboestrol dipropionate in the last weeks before tablet removal, and the tablets in no. 53 were not removed at 60 days but left for the longer interval.

(j) In the other subcutaneously implanted heifers (29, 30, 32, 34, 50-52, 56-58, 121 and 128-136) treatment was continued until the peak yield seemed to have been reached; those not milking satisfactorily were given some additional treatment.

(k) The effect of postponing the start of milking—on which a little information had been given by nos. 1-4 and 27 and 28—was to have been observed on nos. 125 and 137-139; however, in only one of these did the udder fill out as it does in those which come into full secretion and they were transferred to group (j).

(l) Other implanted cows (33, 37-42, 61, 84, 113-115, 126 and 127) were treated on the same lines as the heifers of group (j). Listed with the cows in Table 1 are two heifers given a second treatment when the first had caused udder development but no great volume of secretion.

(m) Two cows, nos. 62 and 63, were not implanted and came into milk after a series of injections; with these the heifer N similarly treated can conveniently be grouped, since the udder had developed before treatment.

### *Ovarian changes*

It is possible in the cow to follow ovarian cyclic changes by rectal palpation; the corpus luteum is distinguishable from a follicle by the feel when gentle pressure is applied and usually by irregularity of shape, the ovulation point being distinguishable. At the 2nd or 3rd day after ovulation the corpus luteum is felt as a soft area on the surface of the ovary, growing out as a fleshy body which becomes firmer and at the end of the cycle (about 21 days) becomes smaller and very hard and sinks back into the ovary. In case of doubt more severe pressure may be applied when the follicle ruptures and collapses, whereas the corpus luteum splits in two, or if pressure is applied in the right way, leaves the ovary and is left between the fingers of the operator.

Distinction between a normal follicle and a cyst is not so easily made. In the case of untreated sterile animals there is usually a history of either absence of, irregularity of, or very frequent oestrus; in addition, there may be signs such as elevation of the tail-head. These things can be no guide with oestrogen-treated animals. It is not normal for a large follicle to remain without ovulating for much more than 24 hr. in an animal which has no active corpus luteum; neither is it normal for there to be a number of fair-sized follicles in each ovary; such follicles will here be spoken of as cysts. Cysts which have existed for some time may be recognized by their tough, thick wall; abnormally large size also may sometimes distinguish a cyst. In the typical case of a cow with a cyst, rupture of the cyst is followed by the development of a follicle which does not ovulate, but which, if broken soon enough by hand, will form a corpus luteum [Hammond, 1939]. This follicle, under the definition above, is a cyst as soon as it has attained a fair size, although, in possessing luteinizing potentialities for a time after this, it might be considered a normal follicle; since this property cannot be recognized at palpation, the former criterion must of necessity be adopted in these circumstances.

The ovarian changes were not followed in the cows 12-16; this group contained the two animals given the lowest doses so that the course of ovarian activity in these would have been of particular interest. Observations have been made on all the other animals. Thirteen normal animals have been killed with a tablet implant still present and the ovaries inspected; in none of these were there any follicles other than very small ones, under 2-3 mm. in diameter (nos. 1, 2, 4, 6, 7, 27, 28, 93, 100, 116, 124, 126 and 138). In all seven cases (nos. 3, 17, 18, 19, 64, 110 and 115) from which tablets had not been removed, but in which none could be found when the animals were killed—probably having been completely absorbed—there was either a large follicle, a cyst, or a corpus luteum and a medium-sized follicle. In addition, when no. 11 was examined at tablet removal 55 days after implanting there was a large cyst in one ovary: the capsule which had formed round the tablets was dissected out, but when opened no tablets were found in it.

With one exception all those animals known to have had a normal cycle at the time of implantation, and examined before tablet removal, had also no palpable

follicle. The exception is no. 89, in which the great majority of the tablets were very early either absorbed completely or else extruded; the few remaining tablets were being absorbed at the rate of 1.7 mg. a day. A small cyst was present in the last month of the treatment period in this animal.

Nos. 33, 61, 116, 124 and 128 had cysts at the time of implantation and no. 106 (after a previous implant) had what may or may not have been a normal follicle. In nos. 116 and 128 the cysts were ruptured at the time of implantation; if any further cysts were formed, they must soon have subsided again. In no. 106 a follicle or cyst was present when the tablets were removed. Nos. 33 and 61 had several cysts each; in no. 61 all except one subsided fairly quickly and this disappeared after 6 weeks; no. 33 reacted similarly except that two tough cysts remained after 2 months. These were then punctured and the ovaries thereafter remained in the anoestrous condition, as did those of no. 124 in which the single cyst was ruptured after a month.

In no. 21 a cyst was ruptured 7 days before implantation, another had been broken a month before; at the time of implanting a third was ruptured and this formed a corpus luteum—recognizable by the transverse scar in place of the usual ovulation point. Whether in the first few days of an implant a follicle can ripen and ovulate is uncertain; there have been cases in which at implantation a large follicle has been ruptured and there was then no active corpus luteum palpable, and in which later a corpus luteum with normal ovulation point was found. Since there is often a large follicle a day after ovulation, at which stage the developing corpus cannot be recognized, this evidence is not conclusive.

There is no reason to suppose that the period of function of the cyclic corpus luteum was ever shortened by an implant; this cannot, however, be established with the available data—dates of oestrus previous to implantation being not often known and almost daily examinations needed to determine the time at which the corpus luteum regressed. In five cases (nos. 2, 21, 67, 69 and 119) it has certainly persisted for longer than normal, and in no. 93 it very probably did so, but this animal was not examined until some 9 weeks after implantation, by which time the ovaries were quite inactive.

In no. 2 the corpus luteum was present at 55 days when the animal was killed. In nos. 67 and 119 the corpus luteum was expressed from the ovary at 67 and 32 days respectively. In no. 21 it died out after about 6 weeks and in no. 69 was still large and fleshy at 102 days when the tablets were removed. Nos. 2 and 21 were implanted at, or soon after, ovulation, but in nos. 67, 69 and 119 the corpus luteum was full sized and fleshy, probably, that is, 7–16 days old. The dose range is wide, 3.7 mg./diem for no. 2 to 23.8 mg. for no. 69. Surprisingly, in no. 117, 2 months pregnant when implanted with over 1 g. of stilboestrol, the course of pregnancy continued uninterrupted, so that the corpus luteum was probably unaffected.

A small number of observations have been made on the effect of gonadotrophins on the oestrogen-suppressed ovary. 5000 or 6000 i.u. of pregnant-mare-serum extract were given in the following cases: no. 17, 10 weeks after implanting; no. 19, 29 weeks after implanting; and to no. 26, 9 weeks after the first implant, and again in the 27th week when the tablets had been removed but the ovaries remained inactive.

In no. 17 several follicles developed and one ovulated to form a corpus luteum which lasted about 3 weeks. There was no follicular response in no. 19 and in no. 26 one small follicle developed on each occasion. The first time the follicle was accidentally ruptured a week after the serum gonadotrophin was given; the following day 5000 i.u. of chorionic gonadotrophin were administered, but no detectable further changes followed. Chorionic gonadotrophin was also given to nos. 20 and 57 in similar amounts, again without any palpable change occurring. The reaction to relatively high dosage of serum gonadotrophin was very slight in three of these four cases; however, similar response is sometimes met with in normally anoestrous heifers.

After removal of the tablet implant ovarian activity very soon restarts; thus in no. 141 there were two large follicles 5 days after removal of the tablets, and in no. 54 ovulation occurred within a week, but these are instances of rather outstanding rapidity. In some cases ovulation has succeeded follicle development almost immediately, while in others there has been an intermediate phase of cystic follicles which has lasted sometimes for over a month. It has been the object to observe only and not to interfere with the process of recovery after tablet removal; however, in several cases these cystic follicles have been broken, but luteinization of these has not been noticed. In five cases, nos. 26, 36, 80, 83 and 86, the cystic condition has continued and there has been no recovery without further treatment.

No. 26 was also remarkable in that follicle growth did not start until 8 weeks after the tablets had been removed; the interval of quiescence in no. 20, treatment of which was long continued, was a full month, again well beyond the usual interval. It is not absolutely certain that all tablets were completely removed in these cases. In heifer 25 two tablets separately encapsulated—from which absorption was proceeding at the rate of 0.7 mg./day—were not removed until 6 weeks after the remainder. The ovaries remained inactive during this period, but within a week of these two tablets being removed a large follicle appeared.

Examinations after tablet removal have been neither sufficiently frequent nor regular for figures to be presented on the point, but it has seemed that the cystic follicle phase of recovery was more marked both with longer duration of treatment and higher dosage level; if so, it might perhaps be due to liberation of administered oestrogen from fat depots in which it had become dissolved during the implant period. Another possibility is that the cystic phase is caused by small chips of tablets not removed; but it is difficult to imagine this can have occurred in all the cases observed.

In no less than eight instances double ovulations have been observed (nos. 20, 21, 29, 49, 66, 96, 108 and 118) after tablet removal, the last six within the first 3 months. In no. 20 it was 6 months, and in no. 21 it was 5 months, after tablet removal; nos. 21 and 96 were in calf and have since produced twin calves at term. The normal frequency of double ovulations is not known, but there is no reason to suppose that it differs greatly from that for twin pregnancies, which may be taken at one in eighty. Twinning is less common in heifers, so one in sixty should be an exaggerated estimate for double ovulations. Most of the heifers were not again examined in the first few months after tablet removal, once a corpus luteum had been found, unless to confirm pregnancy; there thus seems little doubt that an increased frequency of double



ovulations in the first few cycles is a feature of the recovery period after oestrogen implants.

### *Uterus and vagina*

In the group of heifers 1-5 the uteruses and cervix of the control weighed 240 g. and those of the treated animals 161, 165, 182 and 191 g., the last of these being that in which the corpus luteum persisted. The response of the uterus in cattle is thus very different from that found in most laboratory animals. The most characteristic feature of the uteruses in implanted animals was the loss of tone, against which the persistent corpus luteum offered at least a measure of protection. Nos. 17, 18 and 19 were killed after a long period of implantation, tablets being eventually absorbed; ovarian activity reappeared in no. 17 first and in no. 19 only in the last fortnight before killing. They were killed at the same time. The uterus in no. 17 was of normal size and tone, in no. 18 the uterus was flabby and there was some clear fluid in the uterine lumen and no. 19 was similar, except that there was much more fluid.

In the case of some of those animals with 'intraperitoneal' implants, such as nos. 84 and 101, in which there has probably been a short period with relatively high absorption followed by a long period of low dosage, the tone of the uterus had become normal before there was any sign of activity in the ovary. After tablet implants of a 100 days' duration or less the uterine tone has been largely recovered within a fortnight of removal.

In the heifers with persistent hymens or no cervix the volume of accumulated fluid in vagina or uterine horn has increased during treatment and decreased after its cessation. Two cows (114 and 115) in which there were mummified foetuses expelled these during treatment. This happened on the 7th day in no. 114—which choked itself swallowing the cleansings—and at some time unknown in the other, the uterus of which at killing was nearly normal non-pregnant size.

The cervix does not relax during treatment as it does at oestrus, neither is there a marked flow of mucus. Cervical mucus is small in amount and it is relatively thick, but not of the rubbery consistency of late pregnancy. The vulva may appear slightly oedematous and there is some growth of the vagina; but this development and increased lumen diameter is not so great as occurs during the first pregnancy. There has often been a yellow-brown vaginal discharge; search for pathogenic organisms met with no success.

### *Conformation and behaviour*

Following tablet implanting there has often been a loss in condition in the first weeks of treatment and delay in shedding the rough winter coat has been noted. Relaxation of the pelvic ligaments, giving a 'fallen-in' appearance at the side of the tail, occurs consistently and this is followed by elevation of the tail-head. When there has been a persistent corpus luteum these changes did not occur until after it ceased to function.

There has been much variation in the extent of this lifting of the tail-head; in some cases it has been pronounced but in others unnoticeable, and the degree shows no relation to the oestrogen dosage. In some of the heifers given more prolonged treatment there has been an audible creaking of bones on movement in the pelvic region. This and elevation of the tail are sometimes present in cows with cystic ovaries.

After removal of the implant these changes are reversed; but the tail-head does not return to its normal position nearly so rapidly as after calving and, as might have been expected, it has remained up when there has been no return to a normal cycle and the ovaries have remained cystic.

Relaxation of the pelvic ligaments, together with frequent mounting by some treated animals, has probably been responsible for the pelvic fractures which have occurred. In one case the sacrum was fractured and in another probably the rim of the acetabulum; in the majority of cases the wing of one ileum has been knocked down. Healing does not seem to have been impeded by continuance of treatment. In nos. 115, 126 and 139 there was a fracture on each side, and no. 70 did not heal well; these had to be killed, and no. 93 was also slaughtered. Among the forty-eight heifers of the 'planned experiment' there were five fractures; however, not very much meaning attaches to such figures without detailed consideration of the management of all the animals; poor feeding seems likely to have been an important contributory cause. Incidence of haematomas has been above the average in treated animals; increased activity could account for this, but conceivably there is also an effect on fragility of blood vessels or clotting.

It is not always easy to characterize a particular type of behaviour as either male or female. In the cow, besides willingness to accept the bull, restlessness and mounting and riding other cows, or sometimes being ridden by them, are indications of heat. The ewe in heat will stand when prodded by the head or forefoot of the ram, and if heat is intense will seek out the ram and lick his face; but there is no jumping of other animals.

McKenzie & Terrill [1937], in ewes given daily oestrogen injections, after a period observed mounting and also teasing of other ewes in the manner of a ram. Ewes given 1 mg. of stilboestrol dipropionate early in heat have been seen to ride other oestrous ewes, and an ewe lamb implanted with oestradiol would tease and ride ewes, and did so a year after the tablets had been removed [Hammond, Hammond & Parkes, unpublished]; it was found when killed to have very large cystic ovaries. In these cases it is difficult to consider teasing as anything but typically male, but mounting and riding can be taken as representing no more than an extension of the normal behaviour pattern to include what is a usual feature in a related species.

There has been considerable diversity in the behaviour of the implanted cows and heifers. In the majority of cases heat has occurred at irregular intervals, with a frequency of from once or twice in the whole period up to two or three times a week; often treated animals kept in a separate group were quite quiet among themselves. By accident or misunderstanding nos. 19, 65, 83, 85 and 87 were all taken to the bull and accepted service; in contrast, no. 39, which had been run with the bull for company, was left with him during treatment, and so far as is known was never on heat or attracted the interest of the bull.

In those animals in which the corpus luteum persisted there were no signs of bulling and they remained quiet so long as it continued to function. No. 141 ovulated at about the time of implantation and was quiet until 20 days later, at which time the corpus luteum had become very hard, when it was restless, but showed no other sign of heat, and so continued nearly every day until removal of the implant. Others of the unusual cases were no. 61, which roared rather in the manner of a bull and

no. 101, a heifer, anatomically normal, which had never been seen on heat. It was bulling 8 days after implantation; later reports on behaviour, in the words of the owner, were: 'Has changed from being timid to aggressive, and now boss of nearly all the herd after fighting them', and 'no signs of coming into service, and she is able to detect all other cows and heifers 12-24 hr. before they are in heat'. No. 12 was found useful in picking out from a bunch of heifers those which were on heat; the most characteristically male mannerisms were met with in no. 112, which would put its head down and paw the ground.

After tablet removal irregular oestrous behaviour ceased within the first 3 or 4 days; there do, however, appear to be some after-effects. Heat in many cases has not been observed at the first ovulation, and in four animals (nos. 25, 29, 30, 71), sometimes on more than one occasion, signs of heat have been shown, but they have refused to accept service.

### *Fertility*

Details of fertility after treatment are given in Table 1. It should be remembered that all these were animals which had failed to get in calf. In some cases the reason was clear: that it was anatomically impossible. In those apparently normal on rectal examination there may yet be some impediment—it was not discovered that no. 32 had no lumen to the cervix until it had been served several times. Possibly a few of the heifers were anoestrous during the period in which they were run with the bull; metritis is not common in heifers, nor are cystic ovaries, a condition which rarely clears up without treatment. No. 21 had had cysts and been treated; details of the others are given in the table. Some may have got in calf and aborted early without being noticed; heifers put late to the bull are sometimes difficult to get in calf, but having calved are not thereafter infertile.

Whatever the reason for sterility in those found anatomically normal, it is in individual cases unknown but probably lies amongst those listed. There are in the table thirty-four animals in calf and about 150 services; so that it may be said that, on the whole, the heifers remain less fertile after treatment; it does not show that treatment impairs fertility, or even that it is not beneficial. Only two animals have had sufficient time to have calved twice, so it cannot be said if such animals remain difficult to get in calf, but it has not been so with these two.

Abortion was induced in no. 22 because it developed hydrops amnii; there have, however, been six abortions or premature births at 3 months or later, which is a very high incidence. These may have been due to the previous treatment, but it seems not unlikely that they were due to some unbalance, permanent or temporary, which had been responsible for their failure to get in calf up to the time of treatment.

### *Udder development and nature of secretion*

Udder slices from heifers of group 1-5 are illustrated in Plate 1, figs. 1-3: they were prepared in the way described by Hammond [1927], who gives illustrations of the gland at various stages of pregnancy with which these figures may be compared. The great extension of the gland in the treated animals, beside that in the control, no. 5, makes quite clear that there has been an increase in the amount of gland and not merely filling out of the mammae with secretion from pre-existent tissue. Histo-

logical examination revealed development after 52 days parallel to that in late heifer first pregnancy [see Hammond, 1927].

The area of a cross-section probably does not give a very accurate measure of the total volume of gland, so that too close comparisons are unwise, but it does seem that the amount of gland is not quite proportional to the dosage; in particular no. 2 with persistent corpus luteum and the lowest oestrogen dosage shows good development. The development in heifers 8, 10, 27, 28, 64 and the freemartin 50 has also been macroscopically, but not histologically, examined. Nos. 8 and 10, which had had low dosage, were similar in the extent of the gland though their secretion had been so different; nos. 27, 28 and 64 had all extensive development—greater than in nos. 1–4. The freemartin secreted only a small volume of colostrum-type milk; there was a considerable amount of tissue, which was very dense, in the centre of the pad of fat and not spread out through it as in the other animals.

The extent of development in the other animals can only be gauged indirectly from the feel and appearance of the udder and the volume of secretion. While full secretion may reasonably be taken as showing adequate udder development, absence of secretion, or a low level of secretion, need not indicate any deficiency of mammary growth.

This point is very clearly brought out by the history of heifer 90, and cases in which a sudden increase in yield was brought about. After 99 days' implantation no. 90 showed practically no visible udder development, but came into milk a month later and gave 2 gal. a day, and, but for rather characteristic markings, was not then recognizably the same animal. Nos. 17, 19 and 20 at first showed practically no secretory activity, had tiny udders and small teats. Later the yields increased to 1 or 2 lb. and the teats were very large and the udder poor; after stilboestrol injection the yield rose rapidly in no. 20 and the udder appeared normally well developed. Hard tissue could be felt in the udder fat pad of nos. 17–20 well before secretion started, and has also been felt in other animals implanted but not actively secreting. Two heifers were subcutaneously implanted, which did not at any time show signs of active secretion and in which the udders and teats remained small; they were nos. 57 and 125. It was not practicable to obtain the udders of these; it is not thought that mammary development in them had not occurred.

The teats of no. 90 were very small at the end of the treatment period and were rather wrinkled; after lactation started they were of normal size. Though oestrogen caused teat growth, distension by secretion and in milking seems to be needed for the extent of growth to become apparent. There are great breed and individual differences in teat size; no obvious relation to dosage level was noticed, nor to length of treatment. Two teats from one side of the udder were cut off and weighed in heifers 1–5; weights were respectively 41, 27, 15, 19, 9 g., so the persistent corpus luteum of no. 2 does not seem to have inhibited teat growth.

Reports on whether the milk was colostrum or otherwise abnormal were asked for from those in charge of all the various animals, and a sample was drawn from the teats of those not milking when they were visited. From nos. 18, 19 and 21–26 samples were taken weekly for 8 weeks, starting 7 weeks after implanting, and a ninth sample 2 months after the last of the weekly series. For the first 4 weeks fat, lactose, ash and total nitrogen were estimated; in the later samples the nitrogen

distribution was also studied. Later, weekly samples, from the start of secretion until 20 weeks after the beginning of treatment, were taken from cows 61, 62 and 63, and from heifers 65-70; in these the nitrogen distribution only was studied. The heifers were fortunately chosen; besides being all under the same management and half each in the 60- and 100-day group ('planned experiment'), two, nos. 67 and 69, had persistent corpora lutea.

Lactation is dealt with in the section following, but, before detailing analysis findings, some outline of its course in these animals is necessary. Details of dosage and some indication of yields are given in Table 1; nos. 22-26, 65, 66, 68 and 70 all came into milk rather more rapidly than the average, no. 25 continued to increase in yield for some time after tablet removal, the remainder were near their peak yield by about 9 weeks. When samples started nos. 18 and 19 were giving respectively about  $1\frac{1}{2}$  lb. and rather less than  $\frac{1}{2}$  lb., in both the yield rose very slowly to 14 and 7 lb.; in no. 21 a persisting corpus luteum had just died out and the yield rose slowly at first, but soon more rapidly. In no. 69 the corpus luteum persisted for the whole period of implant; the yield was very low and only rose—which it did rapidly—after a stilboestrol injection on day 125. In no. 67 a persistent corpus luteum was removed on the 67th day; from the 20th day it produced  $1\frac{1}{2}$ -2 lb. daily until a fortnight after removal of the corpus luteum (except the 2 days after removal when it was 'sick' and there was very little) when yield quickly jumped to  $1\frac{1}{2}$  gal.

The first lot of samples (nos. 18-26) were started too late for the early effects to be observed; ash was normal in all, fat percentage was very erratic, probably because of unskilled sampling. Lactose was a little higher than the average; total nitrogen and casein were also above average, and no change in these was noted following tablet removal in nos. 22 and 23; figures were still high for all cases at the late sample. Total nitrogen was, with low yields, particularly high in the first and second samples from no. 21 and also nos. 18 and 19; when nitrogen distribution was determined no. 21 had become normal, but no 18, and no. 19 more definitely, showed a relatively high globulin which dropped gradually with further samples.

In nos. 65, 66, 68 and 70 nitrogen distribution was from the start of observations (which did not include the first few milkings) normal. In all these animals casein showed a tendency to rise during the whole period; this must have been a seasonal effect and tablet removal did not influence either casein or globulin percentage. In no. 67 the (total protein minus casein) nitrogen was three times the normal while the yield remained low, and remained quite unchanged after the corpus luteum was removed, but dropped to normal when the yield rose; no. 69 was not sampled after the 86th day until full secretion had started; in the first period the globulin was exceedingly high, but was normal after milk secretion started. In the cows casein and globulin were normal for the whole period examined, except that in no. 61 globulin was high in the first sample, which was also the first milking.

In the cows in milk dried off by implants the milk has become colostrum in character at the end; milk from most of the animals has been reported as colostrum in type in the first few milkings, and sometimes for longer; occasionally there has been a little blood in early milkings.

A little thick honey-like or sticky opaque thinner fluid has been found in all animals examined while yet dry after a period of treatment, including nos. 57, 90

and 125, and cows such as 15, 16 and 40. The thick secretion was present in N and no. 128 before treatment was started.

### *Lactation*

Table 1 gives for each animal the average daily yield in pounds for the 4th, 7th, 10th and 15th weeks, the total yields in the first 10 and the succeeding 20 weeks, the period for which milk has been recorded, the average yield at the end of that period and the total amount of milk produced. The weeks are recording periods, the first thus being an interval of from 2 to 7 days. Those animals which have given a commercial lactation and have been dried off either preparatory to calving, or for sale if sterile, are given as 'dry' in the first week in which they were not milked; some lactations are still in progress, and records for others are incomplete, for these the last available figures are given. For those animals in which the yield was not worth while, and which were dried off and disposed of, details of the last week of normal milking are given.

So presented, the figures do not perhaps give an immediate picture of the form of the lactation curve in the various animals. About the shape of the curve, particularly of the rising segments, there has been no great uniformity, and clearly the presentation of individual curves is out of the question with so large a group of animals; in most cases it will be found that the data provided enable the general form of the curves to be determined.

A few of the heifers were giving a gallon a day within a fortnight of implantation, but these were outstanding cases; the time at which secretion commenced, and the level of production at that time, have varied, to a small degree no doubt, depending upon the moment chosen to start milking. In the first few days of active secretion great tenseness of the udder has often been observed—though the actual volume of secretion may have been relatively small. Perhaps this has been because secretion has started before relaxation of connective, or displacement of fatty, tissue has occurred to give storage capacity to the udder.

The volume of secretion has increased sometimes at a slow steady rate after an initial sharp rise, sometimes similarly with a low level and shallow gradient preceding the sharp rise; in other cases there have been a series of small rises with intermediate plateaux. After tablet removal there has often been a further slight rise, but not always. No. 25 was exceptional in that the yield continued to rise steadily for 3 months after tablet removal until it reached 3 gal. daily, twice the amount it was giving when the implant was removed; seasonal changes are, of course, responsible for considerable fluctuations in yield.

In connexion with the type of secretion mention has already been made of the yields of some of those heifers having persistent corpora lutea; while the corpus luteum was functioning the level of secretion has been 2 lb. a day or less (nos. 21, 67, 119) or almost nil (nos. 69, ?93). After cessation of function or removal of the corpus luteum there have been differences in the way in which yield has increased. In nos. 21 and 119 it started to rise almost at once, in neither very rapidly; in no. 67 there was a fortnight's delay and then a rapid rise. It is not known when the corpus luteum died out in no. 69; it may have still been present when the stilboestrol injection was given which was followed by the initiation of secretion. If there was

in fact a persistent corpus luteum in no. 93 then secretion started only very slowly after cessation of luteal function. That mammary development is possible without luteal secretion is clearly shown by the induction of good secretion in a heifer with cysts, such as no. 121, and even more certainly by secretion from nos. 51 and 52, which were probably freemartins, and certainly had small inactive gonads; nevertheless, the udder development shown by no. 2, and the ultimately better yield of no. 21, in comparison with those of nos. 22-24, suggests that the corpus luteum may augment oestrogen-induced development; however, the performances of nos. 67, 69 and 119, relative to those of other animals in their groups, do not support this supposition.

Though the corpus luteum will apparently prevent the development of secretion under oestrogen treatment, it does not seem to affect the drying off of established lactation; in those cows in milk which were implanted the rate of decline in yield was perhaps more rapid with higher dosage, but the effect began at once and did not attend the dying out of the corpus luteum, nor did the rate of decline seem related to the age of the corpus luteum.

Considering, for the heifers subcutaneously implanted, the effectiveness in terms of milk yield of the various substances, dose levels, and durations of treatment tried, it seems that there is no marked difference between hexoestrol and stilboestrol, and that the dose level, excluding only those very low such as 8 and 10, within the range employed is also unimportant. Of the effect of duration on yield most of the information is supplied by the animals of the 'planned experiment'. Because of variations in rate of onset the best comparison is probably between the yields in the period from the 11th to 30th week; the change between the 10th and 15th weeks, roughly the times of tablet removal in the 60- and 100-day groups, also bears on the point.

The group in which the tablets were removed after 60 days was upon the whole rather better than the other at a stage when the tablets had been removed in neither group; this advantage was well maintained over the 11th to 30th-week period. The change in yield in the 10th to 15th weeks is, upon the average, a slight decline in the 60-day group, and a rather larger rise in the 100-day group. Whereas in the animals with shorter treatment yield has altered only by small amounts, nearly all under 2 lb., in the other the deviations are larger, and, but for gains of 1 gal. in no. 55 and 15 lb. in no. 67, there would have been an average loss greater than that in the 60-day group. Since these two animals received supplementary treatment (injection and corpus-luteum removal), which is likely to have been as effective at an earlier period, it seems that continuation of treatment beyond 60 days is undesirable, except perhaps with animals giving a poor yield, though an adverse effect, if any, on milk yield is probably indirect, through falling off in the condition of the animal; it will usually be desirable to get the animal in calf as soon as possible, and on this count the shorter treatment period will obviously be preferred.

Upon treatment for less than 60 days there is little data, except with the intraperitoneal implants which are complicated with changing and unknown dosage rates; in no. 95 early tablet extrusion seems to have been responsible for poor milk yield. There is probably a limiting growth rate for gland tissue, and hence a minimum period required for full development, whatever the dosage employed; for periods above this

minimum different dosages, up to that giving the maximum, should require different periods of treatment for full development. The initial amount of gland must also be important, but comparison of the udder tissue in no. 64 with that in nos. 8 and 10 suggests the dosage for most rapid development to be well above 2 mg. a day.

Except in connexion with persistent corpora lutea, and on milk composition, little has yet been said on the results obtained in cows, and upon those heifers giving unsatisfactory yields. In determining whether an animal is milking to the extent of which it is capable comparison can be made with the records of previous lactations; this cannot be done with heifers, though records for a subsequent lactation may later become available—as in some cases they have (see Table 2). In fact, with the cows, excepting possibly no. 38, such reference is unnecessary; the yields have been much below those for a normal lactation.

Table 2. *Lactations of treated heifers after first calving*

No.	First lactation: after implantation			Second lactation: after normal calving		
	Week first dry	Total yield	Yield in weeks 11-30	Weeks in milk	Yield	Remarks
9	40	2631	1725	12	2196	—
11	63	5470	1719	5	1381	—
21	71	8203	3551	16½	3686	—
22	55	5750	2744	38	2885	Abortion induced (hydrops amnii)
23	64	6672	2541	22½	4794	—
25	63	7444	2362	20	3699	—
29	47	4528	2634	8	2852	—
30	47	4821	2870	6	2055	—
45	(57)	4777	2188	16	2838	Aborted 213 days; no dry period
46	46	4090	2344	18	3960	—
68	57	4142	1779	9	1470	—
85	49	6195	3669	12	2758	—

With tablet implants there seem to be three stages of secretion, though with no precise boundaries between them. What may be called stage 1 is represented by such animals as nos. 90 and 125, in which there has been no secretion other than a trace of thick globulin secretion; cows such as no. 33, which failed to come into milk, and perhaps persistent corpus luteum cases such as no. 69, in which there was very low secretion, may also be considered as falling within this category.

In stage 2 there is some secretion though not proportionate to the amount of udder tissue; secretion is sometimes unequally distributed between the quarters of the udder; for example, in no. 78 at one time nearly all the milk came from one quarter. Into this group all the cows seem to fall, also such heifers as 44, 54, 141. It can be considered as derived from stage 1 immediately, as in the animals mentioned, or after some time, as in nos. 17 and 20.

The third stage, of full lactation, may be reached directly from the first, either immediately, as in nos. 22, 65 and many others, or after an interval, as in no. 90 when secretion only commenced a month after tablet removal; or the second stage may be intermediate, as in those cases where the yield has risen in steps soon after implantation or as in no. 21 after a corpus luteum has died out. or no. 137 where yield rose suddenly 3 weeks after tablet removal.



Apart from such changes in yield which have occurred spontaneously, or those described following removal of the corpus luteum, a jump in yield has sometimes been brought about after injection of stilboestrol dipropionate, stilboestrol, or a mixture of equal parts of these two substances, subcutaneously in oil, in some cases during the implant period, in others after tablet removal.

A series of injections have been given to heifers 17, 20, 43, 53 and 55 before the tablets were removed; the injections were at roughly weekly intervals and the dose usually 100 mg. of stilboestrol dipropionate. The unimplanted cow 62, after an initial dose of 500 mg. of stilboestrol, had two-weekly injections of 100 mg. before secretion started, and 12 weeks later 100 mg. each of stilboestrol and its dipropionate; this was without influence on the yield. The heifer N, besides the amounts shown in Table 1, which were given at intervals of 6 and 3 days, 12 days after the third injection was given 15 g. of triphenylchloroethylene.

N was overdue to calve, had thick honey-like fluid in the teats, the uterus was dragged down into the body cavity, the ovaries could not be palpated, neither were cotyledons discernible. It was thought there was a mummified foetus and oestrogen treatment was given in the hope of getting this away, but only an amount of mucus was ever seen to be produced; when killed a year later the uterus and contents were in the state of the typical 'white heifer', although the cervix was normal. The udder had filled out at the time of the third injection and milking was started; the yield rose in 9 days to 12 lb. and then more slowly to 17, and the fourth injection had no apparent influence on the rate of increase.

Of the other animals no. 43 showed no effect, no. 53 a small rise possibly due to the second injection, but otherwise no marked change, no. 55 a steady rise from  $\frac{1}{2}$  lb. a day to 13 lb. over a period of 25 days, starting a day or two after the first injection and not seemingly affected by the others.

Nos. 17 and 20 were giving 1 lb. a day or less 4 months after implanting when the injections were started. After the first injection to no. 20 a steady rise started, unaffected by the next four injections, broken at the sixth by a drop of 4 lb., but rising again immediately after the last, to reach 20 lb. in 30 days. In no. 17 the udder had filled out and was very tense at the time of the second injection; yield then rose to 6 lb., but declined very slightly with a further five injections.

Single injections of stilboestrol, or equal parts of stilboestrol and the dipropionate, in amounts of 200-500 mg., have been given before tablet removal (or presumed complete absorption) to the following cows and heifers: 19 (twice), 29, 33, 37 (two a week apart), 40 (twice), 42 (two 5 days apart), 56, 57 (twice), 61, 69 (in presence of corpus luteum), 90, 99, 109, 112, 116, 122, 123 (twice), 124, 125, 134, 135, 136, 137 and 139. These injections were all given at least 7 weeks after implantation; no. 56 was giving about 12 lb., no. 61 5 lb., the rest were either dry or giving 3 lb. or less.

Except in the two following cases these injections were without effect on the yield. No. 29 was giving about 2 lb.; 4 days after injection the yield started to rise steadily and reached over 2 gal. No. 40, a dried-off cow, came into milk 10 days after injection. The time at which secretion has started in cows has been very variable; whether its onset in no. 40 was in any way connected with the injection is not clear.

The dosage from the tablet implants in these animals showing augmented yield with stilboestrol injections has not been unusually low; the effect therefore does not seem to be brought about simply by raising dosage to some threshold value for secretion.

Single injections of large amounts have also been given after tablet removal (or return of ovarian activity) in the following animals: 26, 37, 53, 58, 69, 73, 74, 76, 78, 98 (twice), 99, 104, 108 (twice), 125, 133, 135 and 136. Of these nos. 69, 76, 104, 125 were dry, and nos. 108, 135, 136 giving 2 lb. or less; the remainder were giving 5-10 lb. There was no effect except in these instances following. No. 37 rose from 8 to 25 lb.; there are signs of a gentle rise in the 4 days preceding the injection, much accelerated from the day after the injection was given. In no. 69, which had had a persistent corpus luteum, the udder filled out and it was first milked 4 days later, rising in a week to  $1\frac{1}{2}$  gal. Nos. 108, after the second injection, 133, 135 and 136 all rose shortly to give amounts of  $1\frac{1}{2}$ -2 gal. Nos. 133, 135 and 136 were all increasing slowly before injection, and no. 134—in the same group—had risen spontaneously at that time to 9 lb. and continued to reach 2 gal. without any supplementary treatment. Also, large increases have occurred without treatment in other cases after tablet removal (nos. 90, 109, 122, 137 and 139), and it is not certain that the instances detailed can be ascribed to the injections.

It is particularly remarkable that in no case has injection caused a decrease in yield; doses of this magnitude given to two cows in normal lactation were followed by sharp declines lasting for over a week, of from 35 to 20 lb., and from 20 to 9 lb., before there was any recovery.

The pregnant-mare serum and chorionic gonadotrophin injections, details of which were given in the section on ovarian changes, had no effect on milk yield; neither had 100 units of pituitrin, given at or after tablet removal (two animals each). Extra implants, after milking had been started, were given to nos. 19 and 25; no. 19 showed a slight increase, no. 25 no apparent effect.

The persistence of yield in those given a particularly long period of treatment, or coming into milk a long time after the beginning of treatment, is of some interest. Heifer 18 received no treatment other than a tablet implant. The tablets had been completely absorbed when it was killed after 10 months of treatment, but they could still be felt through the skin at 8 months, so a long period at relatively steady absorption may be assumed. The yield rose slowly to a peak of about  $1\frac{1}{2}$  gal. after 4 months, declined steadily to 4 lb. at 9 months and then rose slightly.

Heifer 20 had the implant removed after 6 months, the yield having then been raised to 2 gal. after injection. The total production in the last 40 weeks is practically the same as that of heifer 34 in 39 weeks from implantation. Since nos. 34 and 20 were the same breed, about the same age, and had almost the same peak yield, reached at about the same time (June, July), a fairer comparison could hardly be hoped for: the relatively short period for onset of lactation in no. 34 being counterbalanced by rather worse seasonal conditions for no. 20.

Heifers 108 and 109 only came into production at about 15 and 30 weeks after the beginning of treatment, and almost certainly many weeks after absorption of all or most of the implant; the level of secretion has so far been very well maintained in both cases.

For maximum production a sufficient dry period between lactations is essential; it may be that this rest period can come at any time; on the other hand, and more probably, it may be required during the process of building up the system which will be set in motion at the onset of the fresh lactation. Thus milking of the heifers during the early stages of treatment may prevent the attainment of full milking capacity. The ultimate effect on secretion of delaying milking until some time after the appearance of activity is therefore of some practical importance.

Whether milking can be delayed, apart from possibly improved yields, is also commercially important in that the majority of the heifers have given small quantities in the first few weeks, and therefore during this period the cost of milking exceeds the value of the milk obtained. Nearly all the animals treated have been milked as soon as (if not before) appearance of active secretion. Heifers 1-4, 27 and 28 were not milked at all, and no. 137 was not milked as soon as the udder filled out.

In no. 2 the udder was in the condition of that of a heifer in late pregnancy which had not been milked, clear thick globulin secretion filling teat, cistern and ducts. The five other animals had all milk, or rather colostrum since it had a definitely sticky feel: there is no possibility of nos. 27 and 28 having suckled one another.

The way in which the udder filled out was not watched in the first group; in nos. 27 and 28 it filled out, became very tense and then this tenseness rather decreased; at this stage they were killed. In no. 137 the udder came down a little within a fortnight of implanting, and there was quite a fair development showing at 4 weeks, but it is doubtful whether it ever became tense. An injection at 50 days did not cause any marked change in the udder; at 74 days milking was started, there having been no change in the appearance of the udder. After a week the yield was just over 2 lb. and remained at this level until tablet removal a month later. Three weeks after removal it rose quickly to over 1½ gal.

The udder failed to fill out at all in no. 125, and did so very slowly in nos. 138 and 139, which, had they developed in the usual way, would also have had the start of milking delayed. There is thus no clear evidence on the point in these experiments; but on the whole it seems probable that if milking is postponed too long after the signs of active secretion appear a 'stage 2' lactation may result.

One of the technical difficulties of subcutaneous tablet implantation is that very small tablets are required to ensure total absorption within a reasonable time and that the inevitable clumping together of tablets affects absorption; it is thus necessary to remove the implant. Beside involving a second operation, and one not quite so simple as the implanting, this means that much more oestrogen has to be used than is actually absorbed.

Parkes [personal communication] has found an increased absorption in rats from stilboestrol tablets placed in the peritoneal cavity; the rate in cattle is evidently very great (see next section), and a number of animals have been given intraperitoneal implants to test the possibilities of this route of administration. These trials have already been outlined, and many of the animals used have been referred to in connexion with the various aspects already touched on in those subcutaneously implanted. Further mention will be needed in giving an account of tablet absorption.

The heifers concerned are listed in Table 1 (2a). In place of the duration of implant and absorption rate are given the times at which the ovaries were last palpated and found inactive, and when first a follicle of any size appeared.

In certain cases (nos. 116, 124) in which the ovaries continued long inactive, which did not come into milk, and which were killed, the tablets were found to be all in the flank, between muscle layers. The same is known to have happened in no. 122 which moved violently as the tablets were being inserted; the tablets being afterwards seen through the cannula to be resting on the outside of the peritoneum. It seems rather probable that in the cases of nos. 101, 107, 109, 112 and 123 this may have happened to one or more of the tablets since, relative to the other heifers, return of follicle growth has been so long delayed.

Satisfactory, or relatively satisfactory, lactation together with quick tablet absorption was given by nos. 102, 103, 118, 119 and 120; the case of no. 104 seemed very like that of nos. 57 or 125—development of mammary tissue, and a little thick secretion in the teats, but no filling out of the udder. Nos. 105, 106, 108, 110 and 111 were, with others, not examined again until 62 days after implantation. In each a cyst was found and the udder had in each developed in rather the same way; it had started to fill out after about 3 weeks and then development ceased; no. 111 was milked and gave about 1 lb. a day.

Though the total dose to these heifers was about that received by those subcutaneously implanted, and the duration of treatment in many not very different, the daily absorption at different stages may have been quite dissimilar; this perhaps accounts for the, upon average, lesser effectiveness of intraperitoneal implants.

#### *Tablet absorption*

That no great error is involved in assuming constant absorption from disk-shaped tablets has been shown by Emmens [1941]. Expression of treatment in terms of daily absorption, which enables comparison of those with different durations of treatment, is therefore justifiable.

Because after a time absorption and elimination must reach equilibrium, the rate of absorption measures directly only the sum of metabolism and excretion, not the effective concentration. The relation between dosage and concentration is unlikely to be linear and might tend to a limit, determined by solubilities and affinities of the oestrogen; development of inactivation systems during treatment would result in a lowering of concentration. As these things are unknown the attaching of too much importance to supposed differences in dosage levels between animals is unwise.

Data have been accumulated upon the absorption of several thousand subcutaneously implanted tablets; a brief presentation of some of these may perhaps be of use to other workers. Table 3 gives details of whole tablets, not including those few cases when extrusion of the majority of tablets has occurred. There have also been three cases in which tablets were completely absorbed in a short period: 100 × 50 mg. of hexoestrol in under 59 days, 60 × 67 mg. of stilboestrol in under 55 days and 100 × 14 mg. of stilboestrol in under 52 days.

The tablets are all excipient free, except those of 1 g. and 340–400 mg. which contained 1% of stearic acid. The 340–400 mg. tablets had diameters of 9.5 mm. and thickness about 4 mm. and were flat top and bottom; 25, 50 and 1000 mg.

tablets were roughly the same shape, the diameter of the largest was about 14 mm. These and the 15 mg. tablets, which were almost as thick as those of 25 mg., had convex tops and bottoms. Taking the surface area of 1 g. tablets as unity, those of the others, in descending order, should be about 0.5, 0.14, 0.09, 0.06.

Table 3. *Absorption from groups of subcutaneously implanted tablets*

Rates, mg. absorbed per tablet per day, are based on the difference in average weight of implanted and recovered whole tablets.

Tablet size mg.	No. of tablets in group	Stilboestrol			Hexoestrol		
		No. of implants	Rate		No. of implants	Rate	
			Range	Mean		Range	Mean
15	166	8	0.04-0.08	0.06	8	0.04-0.09	0.06
25	100	7	0.06-0.15	0.086	8	0.09-0.15	0.118
	200	6	0.05-0.12	0.080	8	0.04-0.11	0.082
50	50-100	16	0.10-0.22	0.15	7	0.09-0.16	0.125
	150-300	9	0.07-0.15	0.11	—	—	—
340	8	1	—	0.5	—	—	—
1000	5-20	5	1.33-2.17	1.82	—	—	—

From the figures in Table 3 it appears that the principal factor affecting absorption is the size of the capsule enclosing the tablets, and not the size of tablet used. There is no significant difference between the rates over 60- and 100-day intervals; in fact, for 15 and 25 mg. hexoestrol tablets, taken together, with twelve implants removed at both intervals, the average rate is almost exactly the same; a slight decrease for the longer interval might have been expected through complete absorption of a small number of tablets.

Heifers 17, 18 and 19 each received eighty stilboestrol tablets of 67 mg.; the tablets became scattered and had all been completely absorbed before killing, the minimum absorption must have been 0.22 mg. a day, and so the average initial rate was well above this.

In kidney fat, and between the flank muscles, where tablets have sometimes been accidentally placed in attempted intraperitoneal implants, rates have been rather higher than subcutaneously. 50 mg. stilboestrol tablets have shown the following average rates: about 0.4 mg. (7-day implant), 0.3 mg. (40 days), 0.5 mg. (40 days).

The evidence for rapid intraperitoneal absorption rests largely on ovarian changes, failure to recover tablets from amongst the contents of the abdominal cavity being alone not convincing. A positive item of evidence is supplied by the bull 140, which was killed 8 days after implanting. Five equal 340 mg. tablets were used, four put into the peritoneal cavity and one into the flank, between two muscle layers; this last tablet was absorbed at 1.8 mg. a day. The other four were recovered from between omentum and flank, and a thin layer of omentum had grown over them; absorption was from 5.0 to 8.8 mg., the mean 7.0 mg. a day. This rate is not so great as seems sometimes to have occurred, but the real rate may have been higher, since encapsulation by the caul fat could not have occurred immediately.

In two other cases this sized tablet has been recovered from the flank after 22 weeks; there were three tablets in each case and the rates were 1.0 to 1.1, and 1.4 to 1.6 mg. per tablet per day. The figure given for subcutaneous absorption in

Table 3 is rather too low, for many of the tablets lay together with the flat surfaces opposed, about 60% should be added to correct for the loss in exposed surface.

In Table 1 (2 *a, b*) the times are given for the reappearance of follicular activity in those animals in which an attempt has been made to put tablets in the peritoneal cavity. It has been found in those subcutaneously implanted that there is no follicle growth except with very low dosage; whether, on such a low dose, ovulation can occur has not been determined. The reappearance of follicular activity can therefore reasonably be taken as showing, if not complete absorption of tablets, at least a great decrease in the rate of liberation from them of oestrogen.

If this is not due to reduction in their size through absorption, it must be due to the development of some impeding capsule. If the second supposition be true, and absorption is not rapid then, first, the tablets will remain large—so that it should have been possible to find at least one of the eighty tablets in nos. 64 and 115—and, secondly, the tablets will last a long time, and their effects also, unless the amount liberated be reduced to a negligible level. Lacking any support, this second hypothesis can be discarded, particularly as in many cases the mammary development affords convincing evidence that there has been relatively rapid absorption from the tablets given.

Taking, then, the evidence of the ovaries, it seems likely that in some cases (particularly no. 84) the tablets have not all been intraperitoneal. The range of time for absorption found in the others, for example between nos. 118 and 119, may be due to different situations taken up within the abdomen; also, since the tablets had flat tops, perhaps two may have come to lie together, and thus been in effect a single larger tablet. That this rapid absorption is not due to the disintegration of tablets cannot be certainly shown; the tablets used, however, have been particularly well made, and not at all fragile, and recovered after long implantation in other sites have been still very hard and have shown no cracks. Rapid absorption occurring prior to any such break-up is shown by no. 140:

#### DISCUSSION

In detailing the results observed comment has been made on various particular matters; it may be of interest to compare, with reference to lactation especially, the developments of pregnancy with those occurring during and after an oestrogen implant.

After conception the corpus luteum persists; the excretion of oestrogen, presumably of placental origin, rises slowly, and then more sharply at about 5 months. At this time the thick high-globulin secretion appears in the teats; abortion afterwards is followed by milk secretion, but if the foetus is retained, mummified, the corpus luteum persisting, secretion does not start. The udder of the heifer may relatively early fill out sufficiently to make the teats appear prominent; in the dry cow there is accumulation of pre-colostral secretion, visible in the last month of pregnancy. Towards the end of pregnancy the corpus luteum dies out; shortly before parturition milk secretion commences. For a few weeks the yield rises and then declines slowly, more rapidly at the 5th month of pregnancy, to rise sharply if abortion occurs.

With a tablet implant oestrogen dosage must be rather different, probably rising to a steady level within a few days and perhaps declining slightly thereafter. On implanting a cow in milk the yield declines without any delay for the corpus luteum to die out, and as the yield drops the secretion becomes colostrals. Precolostral or colostrals secretion has been found in all those treated cows and heifers observed which were not actively secreting. Growth of mammary tissue is believed to have occurred in all the treated heifers. Lactation has begun at varying intervals after implantation, and there has been considerable variation in the way in which the volume has risen. In the first few weeks after removal of tablets there has often, but not consistently, been a further slow increase in yield. There has sometimes been no secretion, sometimes secretion at a level much lower than might be expected from the degree of mammary development. A higher proportion of cows than heifers has failed to come into milk, and low secretion has been more common with the cows. There have sometimes been jumps in yield at an interval after tablet removal; injection of large amounts of stilboestrol in oil, before and after tablet removal, in a proportion of cases with low yields has been followed by an increase—depression, as in normal lactation, did not occur.

Persistence of the corpus luteum has not occurred consistently; it has happened with a wide range of dosage, and milk yield has been low so long as the corpus luteum persisted. After it died out, or was removed, during the implantation period, the yield has risen, though not immediately.

There thus seem to be three distinct processes involved in the passage from the virgin heifer or dry cow to the lactating animal. The first of these is growth or activation of the udder, signified by the appearance of a little secretion high in globulin; the second is the initiation, and the third the maintenance of lactation.

Because in the normal animal luteal persistence is associated with a uterine change, pregnancy, pyometra or a retained mummy foetus, it seems possible the oestrogen affects the corpus luteum, when it is affected, through favouring the development of some uterine condition. As it has not occurred more regularly, either in association with a particular dose level or implantation at a definite stage of the cycle, it can hardly be through direct action either on ovary or pituitary.

The luteal secretion perhaps helps mammary growth, but is clearly not essential, as growth seems to have occurred always with the oestrogen treatment. If pituitary hormones are also needed, they must be secreted in response to the oestrogen at the dose levels employed. Initiation of secretion normally occurs at parturition, at about which time, among other changes, there is cessation of luteal action and alteration in oestrogen secretion. Neither a rise nor a fall in oestrogen dosage has necessarily initiated lactation in the treated animals. Continued luteal function has certainly hindered its development, and though removal of the corpus luteum during treatment has been followed by secretion it is not at all clear that this would always happen; that it would happen if the oestrogen had been previously discontinued seems improbable.

Luteal inhibition of initiation might be due to the action of progestin on the mammary cells, or on the pituitary, preventing secretion of an initiating hormone; a third possibility is that luteal maintenance and lactation initiating hormones are alternative; that a single substance has the two actions seems excluded by the absence of immediate secretion after expression of the corpus luteum.

Secretion has been initiated more frequently and more completely in heifers than in cows; age differences do not seem to provide a satisfactory explanation of this fact, which must therefore be related to a difference in the amount and physiological condition of gland tissue. A small amount of secretion-stimulating hormone might more readily be effective if there was only a small amount of gland to act on; this might explain more frequent initiation, but not why more gland becomes active in the heifer than the cow—unless indeed less gland has been activated in the latter.

On maintenance of lactation luteal secretion seems to be without marked effect. Stilboestrol can paradoxically dry off a lactation, or permit one to start and be maintained at the same or a higher daily dosage. That there are stimulatory and inhibitory levels at which the pituitary can be affected is possible, but if so there must be a tremendous power of accommodation. There is little to show whether the drying off is produced through action on the mammary gland or on the pituitary; the absence of any depressant action of large doses, given after tablet removal, to two heifers giving poor yields suggests that the action is on the udder, because presumably the difference between these and normal lactating animals lay in their having a considerable amount of gland developed but not secreting—which may have served to protect the secreting portion from the oestrogen.

#### SUMMARY

1. A hundred and forty cows and heifers have been given oestrogen treatment, nearly all with tablet implants of stilboestrol or hexoestrol. Observations have been made on changes in the ovaries, reproductive tract, udder and behaviour.

2. During treatment follicle growth ceased and occasionally the corpus luteum persisted; after removal of the implant follicle growth began, and ovulating was resumed, sometimes after a transient period of follicular cysts. An increased frequency of double ovulations has been noted.

3. After the corpus luteum died out there have been irregular heats; the frequency and intensity of these has varied greatly, in a few animals behaviour has seemed characteristically male. Relaxation of pelvic ligaments together with repeated jumping has sometimes led to fractures of the pelvis.

4. The heifers used were animals which had failed to get in calf—indeed, in some it was anatomically impossible. After treatment a number of those with normal organs have been served and a large proportion got in calf.

5. Mammary growth in heifers has occurred. Drying off of lactation, and its initiation in cows and heifers, have followed implants. In the presence of a persistent corpus luteum development of secretion is suppressed.

6. Lactation has not resulted in every case, and sometimes the volume of secretion is low, but many animals have given commercial yields—records are not complete, but over 130 tons of milk have been produced.

7. Data are given on rates of tablet absorption; very rapid absorption within the peritoneal cavity has been observed.

8. There is some discussion on the possible mechanisms involved, particularly in connexion with lactation.



Those who have co-operated so generously in making available the animals for experiment, and in keeping the necessary records, are too numerous for individual mention; we here record our deep appreciation. To Drs J. Hammond and A. S. Parkes we are indebted for much valuable advice and interest. Thanks are also due to Dr G. D. Shearer, by whom the milk analyses were performed, and to Mrs A. Shearer for her help in the compilation of records. The tablets were prepared by Messrs Boots. The work has been financed by the Agricultural Research Council.

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## EXPLANATION OF PLATE 1

FIGS. 1-3. Udder slices from heifers killed 52 days after implantation with stilboestrol. No. 5, untreated; no. 4, total dose 420 mg.; no. 2, total dose 190 mg., persistent corpus luteum.

## EXPLANATION OF PLATE 2

FIGS. 4, 5. Heifer 22 at the time of tablet implant, and again 23 days later, at which time she was giving 10 lb. of milk a day.



Fig. 1

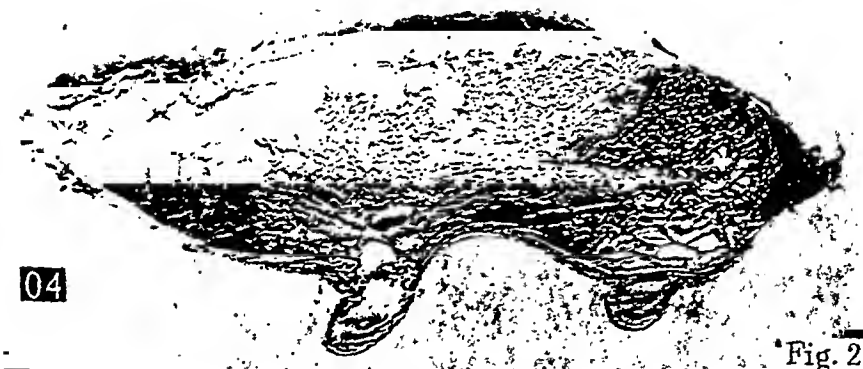


Fig. 2



Fig. 3



FIG. 5.



FIG. 4.

# OESTROGEN EXCRETION IN MILK FROM OESTROGENIZED CATTLE

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(Received 17 December 1943)

Folley and his colleagues [Folley, Scott-Watson & Bottomley, 1940, 1941] have recently shown that copious lactation may be produced in virgin heifers by the administration of diethylstilboestrol. If this discovery is to be used in veterinary practice, it is obviously important to determine whether the milk so produced contains diethylstilboestrol, and if it does, whether the quantities present are liable to have any action on the people drinking the milk. We have not been able to find any reference in the literature to the excretion of oestrogen in normal milk.

The present report is unfortunately incomplete owing to the departure of one of us (S. W. S.) from this Institute. The preliminary results we have obtained, however, do indicate that small amounts of oestrogen are present in the milk and that it is possible to extract this oestrogen and assay it. None of our results has suggested that the amounts of oestrogen present are sufficient to be harmful to the human organism. Preliminary assays of the oestrogen present in the blood and urine of the experimental cattle have also been made.

## MATERIAL AND METHODS

### *Samples tested*

The samples of blood, urine and milk that were tested were supplied by Dr Folley of the National Institute for Research in Dairying, Shinfield, and by Drs Day and Hammond of the School of Agriculture, Cambridge. Particulars of the animals and their treatment are given in Table 1. Some of the milk samples were preserved with benzoic acid.

Table 1. *Samples tested*

Animal	Origin	Oestrogen used	Mode and duration of administration	Wt. of tablets mg.	Daily absorption or dose mg.
Milk:					
26/Jan.	Cambridge	Stilboestrol	Tablet: 6 weeks	200 × 50	31
26/Mar.	"	"	" 3 months	200 × 50	31
21/Mar.	"	"	" 2 months	50 × 50	12.5
24/Mar.	"	"	" 2 months	150 × 50	17
1/Feb.	Shinfield	Hexoestrol	" ?	?	?
† Linnet/Mar.	"	"	" 3 months	8 × 50	0.7
Russet/May	"	"	" 10 days	1000	4
Poppet/May	"	"	" 10 days	1000	2.5
Blood and urine:					
Peasblossom/Mar.	Shinfield	Dienoestrol	Oral	—	24

† Goat.

\* Beit Memorial Research Fellow.

*Continuous ether extraction*

The milk (usually 2 l.) was extracted with ether in a continuous liquid extractor for a minimum of 24 hr., the ether solution being changed every 3-4 hr. to minimize the difficulties caused by emulsification. The ether solutions were combined and any emulsion present was broken down by centrifugation. The solution was then evaporated to a convenient bulk (600-700 ml.), extracted twice with 50 ml. portions of saturated  $\text{NaHCO}_3$  solution, five times with 50 ml. of 2N NaOH solution, and finally washed twice with 50 ml. of water. The NaOH and aqueous extracts were combined and made acid to litmus with concentrated HCl. This acidified solution was extracted four times with 200 ml. of ether. The ether extracts were combined, washed twice with 50 ml. of water, dried over anhydrous sodium sulphate and then evaporated to dryness. The dry residue was dissolved in sesame oil for biological assay.

This method took 4-5 days in all, and there was usually some difficulty with the emulsions formed during the continuous extraction. It proved, however, to be the most satisfactory method adopted and has particular advantages where large volumes of milk have to be handled.

*Acetone precipitation method*

The milk was stirred into 3 vol. of acetone and allowed to stand, and the precipitate was filtered off. The precipitate was washed three times with acetone and then extracted twice with ether. The acetone washings were combined with the original filtrate and the acetone removed by distillation; the resulting aqueous solution was extracted four times with  $\frac{1}{2}$  vol. of ether. These ether extracts were combined with the ether extract of the precipitate and evaporated to 600 ml. The subsequent extraction of the phenolic fraction was carried out as in the continuous ether extraction method.

This method was particularly suitable for smaller quantities of milk and the extraction could be completed in one day. There was no difficulty created at any stage by emulsification.

*Acid precipitation method*

A method of extraction was evolved in which material was precipitated by adjustment of the pH to 4 by addition of HCl; the precipitate was treated similarly to the precipitate in the acetone method and the filtrate by continuous ether extraction. The precipitate tends to be gelatinous and emulsification was still present during the continuous ether extraction. The method therefore had no practical advantages and was abandoned.

*Extraction of blood*

The sample of blood was extracted in exactly the same way as a sample of milk by the acetone precipitation method.

*Urine*

So much oestrogen was excreted in the urine that no extraction was necessary—in fact the urine had to be diluted for testing purposes.

## BIOLOGICAL ASSAY

*Direct testing of milk*

An attempt was made to estimate the oestrogen in the milk by feeding it to ovariectomized rats, either by replacing their drinking water with the milk or by feeding them on an all-milk diet. Details are given with the results below.

*Subcutaneous injection of extracts*

Extracts of experimental milk, and of milk to which known amounts of oestrogen had been added, were dissolved in the requisite volume of sesame oil, and injected subcutaneously into ovariectomized rats by the procedure usually adopted in this Institute, viz. six twice-daily injections of 0.5 ml. were given and the vaginae of the rats smeared twice on the third, fourth and fifth days.

*Intravaginal administration of extracts*

Groups of ovariectomized rats were given two intravaginal applications of the extract dissolved in sesame oil. The applications were each of 0.01 ml. and given on successive days. The vaginae were smeared three daily on the third and fourth days.

*Criterion of vaginal response*

A vaginal smear was considered to indicate a positive response when there was a complete absence of leucocytes from the smear, that is to say, both pro-oestrus and oestrus were taken as indicating oestrogenic activity in the injected sample.

## RESULTS

*Replacing drinking water with milk*

Groups of nine to ten ovariectomized rats fed on a made-up dry diet (Thorley's Rat Cake) had their drinking water replaced for 5-day periods by experimental milk or by normal milk to which known amounts of diethylstilboestrol had been added. Vaginal smears were made twice daily.

Table 2. *Response of ovariectomized rats to various milk samples substituted for their drinking water*

Sample	Stilboestrol added $\mu\text{g./pint}$	5-day milk intake ml./rat	Total stilboestrol intake $\mu\text{g.}$	% positive response
26 (26 Jan.)	—	125	—	0
26 (19 Mar.)	—	200	—	10
Control	28.5	125	6.3	100
"	11.4	115	2.3	67
"	8.5	125	1.9	10
"	5.7	125	1.3	0

The results given in Table 2 show that the method of assay is only satisfactory for milk samples containing 9–12  $\mu\text{g.}$  of diethylstilboestrol per pint or more. These concentrations were not found in the samples of milk tested either by this or other methods. The 10% response obtained in the second sample from 26 is not considered to be significant. The positive response was only obtained in a single smear from one rat and none of the cells was cornified.

*Feeding rats on an all-milk diet*

Dr S. K. Kon suggested that the milk intake could be increased if the rats were fed entirely on milk. A group of ten ovariectomized rats were therefore maintained on an all-milk diet with added copper, manganese, and iron salts [cf. Elvehjem, Hart, Jackson & Weckel, 1934]. After 3 weeks on this diet, the daily intake of each rat was 70–80 ml. of milk. Tests were then carried out on milk containing various amounts of added oestrogen. The oestrogenized milk was fed for 5 days, during which time the vaginæ were smeared twice daily. A period of 9 days on normal milk elapsed between successive tests.

Table 3. *Response of a group of ten rats on an all-milk diet to various concentrations of oestrogen added for 5 days*

Date	Oestrogen added	Concentration µg./pint	Total 5-day intake per rat		% positive response
			Milk ml.	Oestrogen µg.	
20/4	Stilboestrol	3.0	400	2.1	60
4/5	"	2.0	350	1.3	10
18/5	Hexoestrol	3.0	355	2.1	8
2/6	"	5.7	385	3.9	10
16/6	Stilboestrol	3.0	300	1.6	0

With the increased intake of the rats, the sensitivity of the direct feeding test had been increased nearly fourfold. It was found, however, that the sensitivity of the rats apparently diminished with successive tests as the figures in Table 3 show. For this reason and because the amounts of oestrogen that could be detected were still not small enough to be of value in testing the experimental milk, this method was abandoned. The reason for the loss of sensitivity is not known; it may be due to some dietary deficiency, although the rats appeared quite healthy and gained in weight throughout the period on the diet.

*Assay of milk extracts by subcutaneous injection*

The results of these experiments are given in Table 4.

Table 4. *Assay of milk extracts by subcutaneous injection in ovariectomized rats*

Sample	Extraction method	Hexoestrol added µg./pint	Volume extracted pints	Test dose pints	No. of rats	% response	Estimated oestrogen content µg./pint
Control	Ether	—	8	1½	5	0	—
"	Acetone	—	2	½	5	80	—
"	Ether	1.0	4	¾	5	100	0.6
"	Acetone	2.0	1	½	4	100	2.0
				1½	2	100	
21/Mar.	Ether	—	4	¾	5	0	<0.4
	Acetone	—	1	½	5	100	2.0
24/Mar.	Ether	—	4	¾	5	60	0.5
	Acetone	—	1	½	5	20	1.2
26/Mar.	Acetone	—	1	1½	5	0	<1.0
				½	2	0	
?/Feb.	Ether	—	5	½	5	0	<0.5
Linnet	Acetone	—	3	½	5	0	<0.5

These assays give only approximate values, since the amount of oestrogen was so small that only small groups of rats could be used for the tests. The values to be given to the 100% responses were judged by the time at which the rats came into oestrus after the first injection. The positive result obtained when the control milk to which no oestrogen had been added was extracted by the acetone method is particularly unfortunate. There had apparently been some contamination which is not entirely unexpected when such minute quantities of oestrogen are being tested for in a laboratory engaged in the preparation and investigation of synthetic oestrogens of high potency. It will be noticed that the values given to the acetone method are higher in all cases than those given by the ether method. The recovery of hexoestrol added to normal milk was satisfactory in the two tests made. The results in general indicated that the amounts of oestrogen present in the milk were so small that a more sensitive method of biological assay was necessary. This is the reason for the tests by intravaginal application recorded below.

#### *Assay of blood extract*

The heifer Peasblossom from which the blood was obtained was receiving dienoestrol by mouth in a dose of 24 mg. daily. The extract of this blood prepared by the acetone method was tested by subcutaneous injection into single ovariectomized rats in increasing doses. The lowest dose tested was equivalent to 0.25 ml. of the original blood; the other doses tested were equivalent to 0.5, 1.0 ml. and so on in geometrical progression. The first positive result was given by a dose equivalent to 128 ml. of blood. The remainder of the extract was tested in a dose of 100 ml. equivalent in five rats and gave a 100% response indicating a blood-oestrogen concentration of 5  $\mu$ g. per l. in terms of dienoestrol.

#### *Oestrogen content of urine*

A combined sample of the urine from Peasblossom and Cobweb (a heifer receiving 20 mg. of dienoestrol daily by mouth) was injected by our usual technique into single ovariectomized rats in various dilutions. 0.2 ml. of urine gave a positive result and 0.1 ml. gave a negative result. The oestrogen present in the urine is therefore equivalent to approximately 2000  $\mu$ g. of dienoestrol per litre. This would be about 100% of the dose administered but the figure given is only approximate.

#### *Milk extracts tested by intravaginal application*

Samples of milk from two heifers each with a 1000 mg. tablet of hexoestrol implanted subcutaneously, were extracted by the ether method and the extracts tested by intravaginal application. The results are given below in Table 5.

Table 5. *Intravaginal assay of milk extracts*

Sample	Volume extracted pints	Test dose pints	No. of rats	% response	Hexoestrol content $\mu$ g./pint
Russet	5	$\frac{1}{8}$	10	30	0.02
		$\frac{1}{4}$	10	30	
Poppet	5	$\frac{1}{8}$	10	40	
		$\frac{1}{4}$	5	100	0.01

These results are considerably lower than those obtained in Table 4. Consideration of the latter figures, however, shows that positive responses were only obtained



in one case (24/Mar.) using the ether method of extraction. This heifer was implanted with diethylstilboestrol while the two tested by intravaginal application were implanted with hexoestrol.

#### COMMENT

These exploratory experiments have shown that small amounts of oestrogen are excreted in the milk of heifers treated with synthetic oestrogens. They also show that these small amounts can be extracted and assayed with reasonable accuracy if the method of intravaginal application is adopted. This method has the advantage that esters of the oestrogen are also estimated presumably because they are rapidly hydrolysed locally [Emmens, 1941]. Once accurate dose:response curves have been worked out for the activity of the various synthetic oestrogens by intravaginal application, it should be possible to estimate the oestrogen content on samples of milk containing about  $0.05 \mu\text{g}$ . Thus 5 pints should be an adequate quantity of starting material. Such quantities will necessitate the use of the ether method and consequent longer time to complete the extraction.

The smallness of the number of experiments performed and the approximate nature of the assays do not allow any definite statements as to the actual amounts of oestrogen present in the milk. We tentatively conclude however that the concentration of oestrogen is maximally  $0.5\text{--}1 \mu\text{g./pint}$  and is usually much lower. These amounts cannot unfortunately be estimated by direct feeding experiments, the method of administration most nearly simulating the human consumption of milk. Walker & Stanley [1941] have injected  $200\text{--}1000 \mu\text{g}$ . of diethylstilboestrol dipropionate daily into lactating rats and observed premature vaginal opening in the litters. They conclude that 'large amounts of oestrogen were excreted in the milk'. We think it extremely improbable that there was no leakage from the injection site, and very little of the concentrated solution used for the injection would be required to produce effects in the litters whether absorbed orally or percutaneously. The low concentration of oestrogen found in the blood and the high concentration found in the urine fully support the view that little oestrogen is excreted in the milk.

#### SUMMARY AND CONCLUSIONS

1. Small amounts of hexoestrol ( $1\text{--}2 \mu\text{g./pint}$ ) added to normal milk can be extracted with  $60\text{--}100\%$  recovery.
2. The amounts of oestrogen present in the milk from heifers treated with synthetic oestrogens is too small for assay by direct feeding to rats.
3. Samples of experimental milk were extracted and roughly assayed by subcutaneous injection or intravaginal application in ovariectomized rats. The results obtained using a continuous ether extraction method were:

Heifer/Feb. implanted with hexoestrol:	$< 0.5 \mu\text{g./pint}$
26/Mar. implanted with diethylstilboestrol:	$< 1.0 \mu\text{g./pint}$
21/Mar. implanted with diethylstilboestrol:	$< 0.4 \mu\text{g./pint}$
24/Mar. implanted with diethylstilboestrol:	$0.5 \mu\text{g./pint}$
Linnet/Mar. implanted with hexoestrol:	$< 0.5 \mu\text{g./pint}$ (acetone method)
Poppet/May implanted with hexoestrol:	$0.01 \mu\text{g./pint}$
Russet/May implanted with hexoestrol:	$0.02 \mu\text{g./pint}$

4. Single determinations on the blood and urine of a heifer receiving 20-24 mg. of dienoestrol daily by mouth gave dienoestrol concentrations of  $5\mu\text{g./l.}$  of blood and 2 mg./l. of urine.

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# INDUCTION OF LACTATION IN HEIFERS BY A SINGLE INJECTION OF ESTERS OF DIETHYLSTILBOESTROL

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*(Received 17 December 1943)*

The preceding papers in this series have dealt with the induction of lactation in heifers by the implantation [Folley & Malpress, 1944*a*; Hammond & Day, 1944] or feeding [Folley & Malpress, 1944*b*] of diethylstilboestrol. Both of these methods have disadvantages. Implantation of tablets, though apparently the most effective method, involves a minor surgical operation, a repetition of which may be necessary if the remains of the tablets have to be removed. Oral administration, on the other hand, requires very large dosage and, for some reason, is not very effective. It seems certain that a duration of treatment of not less than several weeks is required to produce the mammary changes leading to lactation, but there is no apparent reason why repeated injection should not produce the required result. Positive results with such a technique have in fact been obtained in various experiments on heifers [e.g. Walker & Stanley, 1940, 1941; Reece, 1943; Kochan, 1943]. However, repeated injection is highly unsuitable for wide-scale practical application; a single injection, on the other hand, if it could be made effective, would have considerable advantages over both implantation and feeding. In this connexion it was natural to consider whether the necessary duration and intensity of treatment could be attained by a single injection of one of the esters of diethylstilboestrol. Miescher, Scholz & Tschopp [1938] showed that the aliphatic esters of oestrone and oestradiol had a duration of action, following subcutaneous injection, proportional to the number of carbon atoms in the acid chain. The intensity of effect, in relation to total dosage, as might be expected, was inversely proportional. Corresponding data for esters of diethylstilboestrol are not abundant, but it seems likely [Dodds, Golberg, Lawson & Robinson, 1938] that a similar correlation holds. Certainly, the rate of absorption of tablets of diethylstilboestrol esters is proportional to the length of the acid chain [Emmens, 1941]. It was decided, therefore, to find out whether lactation could be induced in heifers by a single injection of one or other of the aliphatic esters of diethylstilboestrol. Little is known of the precise duration of treatment required to induce lactation in heifers or of the total dose required. There is even less information about the shape of the optimum dose curve, though the work of Folley [1944] suggests that the implantation of tablets, which seems to be the most effective technique yet found, causes, in bovines, a heavy dosage over a week or so, followed by a much lower dosage for a prolonged period. In the third series of ester experiments an attempt was made to simulate such a dosage gradient by the simultaneous injection of a slowly acting and a rapidly acting ester.

## MATERIAL AND TECHNIQUE

*Heifers*

All the heifers were of the Ayrshire breed, about 15–18 months old at the start of treatment. The two lots of seven heifers used in Exps. I and III were obtained from a first-class commercial herd in Cheshire, and were maintained at the National Institute for Medical Research Farm Laboratories, Mill Hill, during the experiment. The ten used in Exp. II were obtained from various farms in Ayrshire, and were maintained at the Agricultural Research Council Field Station, Compton, during the experiment. They were in rather better condition than the first batch.

*Material injected*

The heifers were treated with one or more of the following esters of diethylstilboestrol: dipropionate, di-*n*-butyrate, dicaprylate, dicaprate, dilaurate, and dipalmitate. The dosages varied from 0.05 to 1.0 g., all calculated in terms of the molecular equivalent of free stilboestrol. It was thought desirable to avoid the injection of large amounts of oily solution, and the substances were therefore suspended in saline at a concentration equivalent to 1 g. of free stilboestrol in 20 ml., a trace of taurocholic acid being added to assist emulsification. These suspensions tended to settle out fairly quickly, but except for the dipalmitate they were quite suitable for injection through a wide bore needle if well shaken immediately before being taken into the syringe. The dipalmitate suspension, which contained 4.2 g. of the ester in 20 ml., was extremely thick and difficult to use. Injection was made under the skin of the neck and the bleb dispersed by massage. Where two esters were injected simultaneously one was given on each side of the neck. Such a double injection would not, of course, be necessary for the regular administration of two esters simultaneously in constant ratio—the two esters could easily be mixed in the same suspension, and the dispersal of the suspension by massage would probably prevent one ester from interfering with the absorption of the other.

*Observations*

The state of the ovaries was ascertained by rectal palpation before and several times after injection. In the Mill Hill experiments, from about 2 weeks after injection, efforts were made regularly to obtain milk from the teats, milking being done twice daily as soon as the daily amount reached 1 lb. or more. Periodic analyses of the milk for fat content and solids non-fat were obtained. The yields shown in the tables and figures are the average daily yields for each 7-day period from the day of injection, given in lb. and oz. In the Compton experiment body weights were also obtained. Observations were made on changes in the shape of the pelvis, occurrence of nymphomania, etc.

*Estimation of urinary oestrogen.* In Exp. I, estimations were made of the oestrogen content of the urine. Each week a 500 ml. sample of urine was obtained from each heifer and extracted according to Method II described by Callow, Callow, Emmens & Stroud [1939]. In this method carbon tetrachloride is used for the initial extraction of the urine, the carbon tetrachloride-soluble fraction afterwards being cleaned up with benzene. In the present work, the further fractionation described by these

authors, designed to separate the oestrogens from the androgens, was not used. The oily residue obtained after evaporation of the benzene extract was tested for oestrogenic activity on ovariectomized mice. From the mouse results the total activity of the extract, in terms of diethylstilboestrol, was calculated and afterwards the activity per litre of original urine.

The results (described on p. 95), though of some interest, did not seem commensurate with the labour involved, and no examination of the urine was made in Exps. II and III.

#### EXPERIMENT I. MILL HILL.

The seven heifers used in Exp. I were treated as shown in Table 1, the injections being made on 24 November 1942.

#### *Milk yield*

The milk yields are shown in Table 1 and Fig. 1. Six of the seven heifers gave a recordable amount of milk at some stage. The following conclusions may be drawn concerning milk yield.

(a) The general level of response was less good than that usually obtained by the implantation technique, but the small size of the animals (600–700 lb.) may have had some effect in this direction. The two heifers receiving the small doses of the long-acting esters responded in a promising manner; 0.25 g. of the dilaurate gave the best response, second place being taken by the same dose of the dipalmitate.

(b) In each instance, 0.25 g. of the dipalmitate, dilaurate, and dibutyrate gave a much greater response than 1.0 g. of the same ester.

(c) Even in these few animals there was some evidence of a relation between the rate of response and the nature of the ester. The most delayed response occurred in the heifer receiving 0.25 g. of the comparatively rapidly acting dipropionate, and the second most delayed in the one receiving the same dose of the dibutyrate. The slowly acting esters, the dilaurate and dipalmitate, caused a quicker response. This apparent paradox is probably due to the fact that the greater intensity of the initial action of the rapidly acting esters as compared with that of the slowly acting ones is likely to cause a more intense initial phase of growth of the mammary gland which in turn will delay the beginning of the secretory phase. The animals receiving the larger dose of the various esters, viz. 11, 18 and 22, exhibited a better development of the teats which showed an average

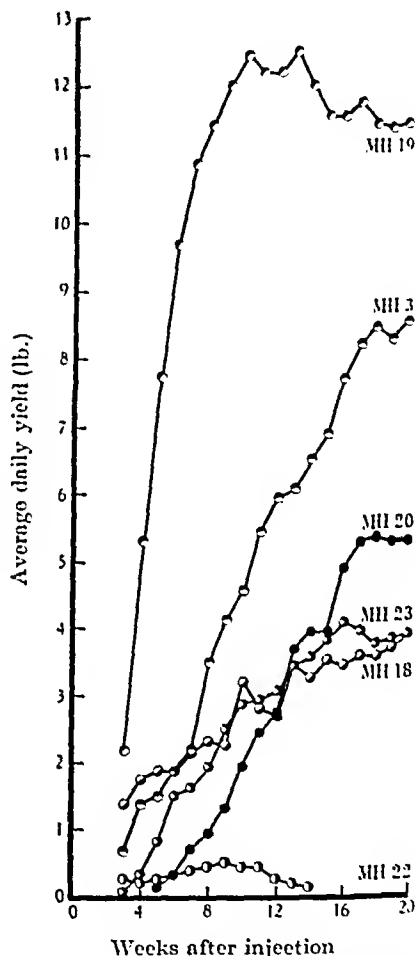


FIG. 1. Exp. I. Milk yields of heifers receiving a single injection of one ester of diethylstilboestrol. MH 20, 0.25 g. of diethylstilboestrol dipropionate. MH 23, 0.25 g. of diethylstilboestrol dibutyrate. MH 22, 1.0 g. of diethylstilboestrol dibutyrate. MH 19, 0.25 g. of diethylstilboestrol dilaurate. MH 18, 1.0 g. of diethylstilboestrol dilaurate. MH 3, 0.25 g. of diethylstilboestrol dipalmitate.

Table 1. *Experiment I. Milk yields*

Ester	Dose g.	No. of heifer	Average daily milk yield for each week after injection																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
			lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.
Dipropionate	0.25	M11 20	—	—	—	—	0 2	0 5	0 11	0 15	1 5	1 15	2 7	2 12	3 11	3 15	3 15	4 14	5 4	5 4
Dibutyrate	0.25	M11 23	—	—	—	0 5	0 13	1 8	1 10	1 15	2 8	2 14	2 15	3 1	3 7	3 9	3 13	4 1	3 15	3 13
	1.0	M11 23	—	—	—	0 3	0 4	0 5	0 6	0 7	0 8	0 7	0 7	0 4	0 3	0 3	—	—	—	—
Dilaurate	0.25	M11 19	—	—	—	5 5	7 12	9 11	10 14	11 7	12 0	12 7	12 3	12 3	12 8	12 0	11 9	11 9	11 13	11 7
	1.0	M11 18	—	—	—	1 12	1 14	1 14	2 1	2 5	2 4	3 3	2 13	2 11	3 7	3 1	3 8	3 7	3 9	3 11
Dipalmitate	0.25	M11 3	—	—	—	1 6	1 8	1 14	2 3	3 8	4 2	4 9	5 7	5 15	6 1	6 8	6 11	7 11	8 3	8 4
	1.0	M11 11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 2. *Experiment I. Milk composition*

No. of heifer		Date									
		14. xii. 43	17. xii. 43	22. xii. 43	30. xii. 43	4. i. 43	13. i. 43	20. i. 43	2. ii. 43	3. iii. 43	
M11 20	Solids non-fat %	—	—	—	10.1	8.9	9.2	8.8	8.6	8.7	
	Fat %	—	—	—	3.3	4.0	5.7	4.3	4.2	4.0	
M11 23	Solids non-fat %	—	6.73	9.7	9.2	9.3	8.5	8.5	8.2	8.1	
	Fat %	—	3.23	2.8	3.8	3.5	5.2	3.0	4.0	4.2	
M11 22	Solids non-fat %	—	10.80	9.7	9.6	10.2	9.4	8.9	8.7	—	
	Fat %	—	3.64	3.2	3.2	3.6	3.0	3.0	3.8	—	
M11 19	Solids non-fat %	9.21	9.74	10.0	9.2	9.8	8.0	8.0	8.9	8.8	
	Fat %	2.76	4.2	6.1	7.0	2.8	4.8	4.5	3.8	3.7	
M11 18	Solids non-fat %	9.85	10.24	9.5	9.7	9.3	8.7	8.3	8.8	8.5	
	Fat %	2.92	4.28	4.8	2.5	4.1	4.7	8.0	4.5	3.5	
M11 3	Solids non-fat %	—	—	9.2	9.7	9.3	9.0	8.9	9.2	8.1	
	Fat %	—	—	9.1	4.1	3.1	5.2	4.1	5.8	4.3	

increase of about half an inch when compared with the three heifers receiving the smaller doses. The size of the teats showed no parallelism with the development of the udder or with the milk yield.

### *Milk composition*

The first milk secreted in all cases was obviously colostrum in type. The duration of this phase varied with the yield and with the individual. Where the yield remained very low, as in MH 22, the milk never acquired a normal appearance. In several of the heifers traces of blood appeared in the milk in the first few days.

The milk from each heifer was sampled and analysed from time to time. The results (Table 2) show that after the first few weeks, the milk, both as regards fat percentage and solids non-fat percentage, was well over legal minima, except in the case of MH 22 already referred to, and of MH 23. In most samples the fat content was definitely high, and in two, perhaps owing to errors of sampling, it was exceptionally high. There was no obvious connexion between the nature of the ester injected and the quality of the milk.

### *Oestrous cycle and condition of ovaries*

The recorded periods of oestrus and the state of the ovaries as examined on four occasions by rectal palpation are shown in Table 3.

Table 3. *Experiment I. Occurrence of oestrus and condition of the ovaries*

No. of heifer	Periods of oestrus	Condition of ovaries			
		5. xii. 42	29. xii. 42	26. i. 43	23. ii. 43
MH 20	4. i. 43 to 7. i. 43	Neg.	Neg.	Neg.	R.O. c.l. L.O. Neg.
MH 23	17. i. 43 to 27. i. 43 (or longer)	R.O. — L.O. s.f.	Neg.	Neg.	R.O. Neg. L.O. c.l.
MH 22	8. xii. 42 to 17. xii. 42 17. i. 43 to 27. i. 43 (or longer) 5. iii. 43 to 8. iii. 43 26. iii. 43 to 29. iii. 43	Neg.	Neg.	Neg.	Neg.
MH 19	Never in season	—	Neg.	Neg.	R.O. c.l. L.O. Neg.
MH 18	25. i. 43 to 2. ii. 43 10. ii. 43 to 23. ii. 43 24. ii. 43 to 2. iii. 43	Neg.	Neg.	Neg.	R.O. small c.l. L.O. Neg.
MH 3	Never in season	R.O. o.c.l.	Neg.	Neg.	Neg.
MH 11	7. i. 43 to 12. i. 43 25. i. 43 to 28. i. 43 (or longer) 2. iii. 43 to 5. iii. 43	R.O. s.f. L.O. Neg.	Neg.	Neg.	R.O. c.l. L.O. Neg.

R.O.=right ovary; L.O.=left ovary; c.l.=corpus luteum; o.c.l.=old corpus luteum;  
Neg.=ovary(ies) quiescent; s.f.=small follicle.

It will be seen that at the first examination, made a few days after injection, the ovaries were all quiescent, and that the only sign of previous activity was an old corpus luteum in MH 3. This quiescence was still found at the third examination made  $7\frac{1}{2}$  weeks later, but at the fourth examination made 13 weeks after injection, five of the seven heifers had active ovaries. It must be supposed, therefore, that the inhibitory effects of the treatment in these five animals had worn off by this

time. It is hard to account for the fact that of the two animals not showing active ovaries after 13 weeks one had received a large dose of the dibutyrate and the other a small dose of the dipalmitate. The heifers were stalled during the whole course of the experiment, and the records of oestrus relate to physical rather than to behavioural symptoms. So far as the records go, however, they do not suggest any undue persistence of heat or of nymphomania. There was slight relaxation of the pelvis in one or two of the heifers, but there were no dislocations or fractures and no sign whatever of prolapse of the vagina or rectum.

#### *Subsequent history*

In order to determine whether the injections had interfered with their capacity to breed, all the heifers, with the exception of MH 11, were sent on 27 April 1943 to the Agricultural Research Council Field Station at Compton for inclusion in one of the experimental herds. As it was not possible to mate them individually, they were allowed to run with a suitable bull for several months. When examined rectally on 26 July 1943 three were found to be pregnant (MH 18, 19, 20) from being served in May. The other three had active ovaries but were non-pregnant. The occurrence of pregnancy did not correlate either with extent of lactation or dosage of diethylstilboestrol.

#### *Excretion of oestrogen*

The results of the assays of the urine samples for oestrogen content are shown in Table 4, which gives the activity of the urine per litre at various times after injection in terms of diethylstilboestrol equivalent. As it was impracticable to measure the

Table 4. *Experiment I. Excretion of oestrogens in the urine*

No. of heifer	Diethylstilboestrol equivalent ( $\mu$ g.) per litre urine						
	26. xi. 42	1. xii. 42	8. xii. 42	14. xii. 42	21. xii. 42	28. xii. 42	4. i. 43
MH 20	—	20	62	28	20	—	—
MH 23	Trace	Trace	396	80	—	—	—
MH 22*	—	196	332	52	54	12	—
MH 19	—	Trace	56	46	—	—	—
MH 18*	—	130	120	96	6	—	—
MH 3	—	Trace	38	Trace	—	—	—
MH 11*	—	120	760	114	116	48	—

\* Heifers receiving the larger (1.0 g.) doses.

volume of urine voided per day, no comparative figures can be given for total oestrogen excretion. The following comments may be made on the table.

(a) For each of the three esters given in two different doses 0.25 and 1.0 g., the larger dose caused the appearance of oestrogen in the urine for a longer period than did the smaller dose.

(b) There was no regular connexion between the nature of the ester and the time during which oestrogen was detected in the urine, or between dosage and the amount appearing in the urine.

(c) If the urine output of these heifers is put at about 10 l. per day, then the maximum daily output of oestrogen detected was equivalent to well under 10 mg., and was mainly less than 1 mg., of diethylstilboestrol. If the active substance present in the urine was actually diethylstilboestrol, as may perhaps be inferred from Stroud's [1939] work on rabbits, then only a small part of the injected oestrogen was recovered in the urine.



Table 5. Experiment II. Milk yields

Ester	Dose G.	No. of heifer	Average daily milk yield for each week after injection													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dibutyrate	0.05	C1	—	—	—	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	
	0.10	C2	—	—	—	0 3	0 4	0 7	0 9	0 9	0 11	0 12	0 13	0 13	0 13	
	0.25	C8	—	—	—	0 3	0 5	0 9	0 7	0 6	0 6	0 9	0 9	0 7	0 6	
Dicaprylate	1.0	C9	—	—	—	0 0½	0 0½	0 0½	Trace	Trace	Trace	Trace	—	—	—	
	0.25	C10	—	—	—	0 0½	0 0½	0 0½	Trace	Trace	Trace	Trace	—	—	—	
	0.25	C10	—	—	—	—	—	0 0½	Trace	Trace	Trace	Trace	—	—	—	
Dilaurate	0.10	C3	—	—	—	0 2	0 3	0 4	0 3	0 2	0 1	0 0½	0 0½	0 0½	Trace	
	0.25	C4	—	—	—	0 2	0 3	0 5	0 7	0 10	0 13	0 13	1 0	1 1	0 15	
	1.0	C5	—	—	—	Trace	Trace	Trace	0 2	0 5	0 10	0 13	1 1	1 3	1 2	
Dipalmitate	0.25	C6	—	—	—	0 3	0 6	0 12	1 8	1 14	2 4	2 11	3 4	3 5	2 14	
	1.0	C7	—	—	—	0 1	0 1	0 11	2 4	3 11	4 8	4 11	4 12	4 8	3 7	

## EXPERIMENT II. COMPTON

The ten heifers used in Exp. II were in rather better condition than those used in Exp. I. The general plan of experiment was as before, the aim being to confirm the results obtained in Exp. I, and to test two further esters, the dicaprylate and the dicaprate. C 1-7 were injected on 18 December 1942 and C 8-10 on 13 January 1943. Observations were made on milk yield, body weight, and condition of ovaries.

*Milk yield*

The results, shown in Table 5, were extremely disappointing. Only the two heifers getting dipalmitate gave a daily average for a week of more than 2 lb. at any stage; six never gave as much as 1 lb. In view of the similarity in the animals used at Compton and at Mill Hill, the difference in response is very difficult to understand, but it may have been influenced by the fact that attempts to obtain milk from the udders were made earlier and more frequently at Mill Hill than at Compton, and that twice-daily milking was the rule. All that can be said about the milk yield in this experiment is that the least bad results were given by the two doses of the longest-acting ester.

*Body weight*

The changes in body weight over the experimental period are shown, for each of the ten heifers, in Table 6. The gains recorded are of a normal order under the existing conditions, and there is nothing to suggest that the treatment had any inhibiting effect on growth.

Table 6. *Experiment II. Body weight*

No. of heifer	21-23 Dec.			29-31 Mar.			Increase lb.
	cwt.	qr.	lb.	cwt.	qr.	lb.	
C 1	5	1	12	6	0	16	88
C 2	6	1	2	7	2	1	139
C 3	6	0	22	7	1	11	129
C 4	7	0	3	8	1	10	147
C 5	4	3	5	6	0	17	152
C 6	6	3	16	7	3	8	104
C 7	6	2	7	7	3	3	136
C 8	5	3	0	7	0	2	142
C 9	6	0	7	7	2	7	178
C 10	5	3	9	7	0	11	142

*Oestrous cycle and condition of ovaries*

Observations made on the Compton heifers are summarized in Table 7. It may be concluded that the effects on the ovaries were generally similar to those observed in Exp. I, though there was a greater tendency towards cyst formation. Symptoms of nymphomania were more marked, possibly because the Compton heifers were allowed out into a yard each day. One heifer (C 1) had to be slaughtered owing to persistent eversion of the vagina. As this animal received only 50 mg. of diethylstilboestrol as dibutyrate it is extremely difficult to believe that the occurrence can have been a result of the treatment. When examined on 26 July 1943, seven of the remaining nine heifers were between 6 and 10 weeks pregnant, the remaining two having active ovaries.

Table 7. *Experiment II. Condition of the ovaries*

No. of heifer	Condition of ovaries										1. vi. 43	Persistent vaginal eversion—heifer slaughtered
	17. xii. 42	31. xii. 42	14. i. 43	27. i. 43	4. ii. 43	25. ii. 43	12. iii. 43	23. iii. 43	5. iv. 43			
C 1	R.O. Neg. L.O. c.l.	Neg.	Neg.	R.O. f. L.O. Neg.	Neg.	R.O. f. L.O. Neg.	R.O. Neg. L.O. f.	Neg.	Neg.	Neg.	Neg.	
C 2	R.O. ? L.O. ?	Neg.	Neg.	Neg.	Neg.	R.O. f. L.O. Neg.	Neg.	Neg.	R.O. Neg. L.O. s.c.l.	R.O. Neg. L.O. Neg.	R.O. c.l. L.O. Neg.	
C 3	R.O. Neg. L.O. c.l.	Neg.	Neg.	R.O. Neg. L.O. f.	Neg.	R.O. f. L.O. 'Cherry' cyst	R.O. 'Walnut' cyst L.O. Neg.	R.O. c.l. L.O. Neg.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	
C 4	R.O. Neg. L.O. c.l.	Neg.	Neg.	Neg.	Neg.	R.O. Neg. L.O. f.	R.O. Neg. L.O. f.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	
C 5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	R.O. c.l. L.O. Neg.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	
C 6	R.O. c.l. L.O. Neg.	Neg.	Neg.	Neg.	Neg.	R.O. Neg. L.O. f.	R.O. s.c.l. L.O. Neg.	Neg.	R.O. c.l. L.O. Neg.	R.O. 'Walnut' cyst	Neg.	
C 7	R.O. Neg. L.O. c.l.	Neg.	Neg.	Neg.	Neg.	R.O. f. L.O. s.c.l.	R.O. f. L.O. Neg.	Neg.	R.O. 'Cherry' cyst L.O. Neg.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	
C 8	R.O. c.l. L.O. Neg.	R.O. c.l. L.O. Neg.	R.O. c.l. L.O. Neg.	R.O. s.c.l. L.O. Neg.	R.O. Neg. L.O. Neg.	R.O. Neg. L.O. f.	R.O. f. L.O. Neg.	R.O. f. L.O. Neg.	R.O. Neg. L.O. 'Cherry' cyst	R.O. Neg. L.O. Neg.	R.O. Neg. L.O. Neg.	
C 9	R.O. c.l. L.O. Neg.	R.O. c.l. L.O. Neg.	R.O. c.l. L.O. Neg.	R.O. Neg. L.O. Neg.	R.O. Neg. L.O. Neg.	R.O. Neg. L.O. f.	R.O. Neg. L.O. Neg.	Neg.	Neg.	R.O. Neg. L.O. Neg.	R.O. Neg. L.O. Neg.	
C 10	R.O. c.l. L.O. Neg.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. c.l.	R.O. Neg. L.O. s.c.l.	R.O. Neg. L.O. Neg.	R.O. Neg. L.O. Neg.	Neg.	R.O. s.c.l. L.O. Neg.	R.O. s.c.l. L.O. Neg.	R.O. c.l. L.O. Neg.	R.O. c.l. L.O. Neg.	

R.O. = right ovary; L.O. = left ovary; c.l. = corpus luteum; s.c.l. = small corpus luteum; Neg. = ovary (ies) quiescent; f. = follicle.

Table 8. *Experiment III. Milk yields*

Ester (1)	Dose g.	Dose No. of heifer	Average daily milk yield for each week after injection																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
			lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	lb. oz.	
Dilaurate	0.25	Free di-ethylstilb-oestrol	0.05	MH 30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
"	0.25	Dipropionate	0.05	MH 25	—	—	0 5	0 5	0 7	0 13	1 14	3 4	4 0	4 10	4 8	5 11	5 0	5 1	5 11	6 4	6 2	5 13
"	0.25	"	0.10	MH 16535	—	—	0 7	2 9	4 1	6 9	7 13	8 11	8 5	9 4	9 5	9 3	9 8	7 12	8 7	8 3	4 8	11 8
"	0.25	"	0.25	MH 23601	—	—	—	—	—	1 0	2 14	5 6	7 4	8 11	10 6	12 5	12 15	12 14	13 6	—	—	—
Dipalmitate	0.25	Free di-ethylstilb-oestrol	0.05	MH 28	—	—	0 3	0 2	0 1	—	—	—	—	—	—	—	—	—	—	—	—	—
"	0.25	Dipropionate	0.05	MH 28	—	0 1	0 7	0 8	1 1	2 3	3 3	4 0	4 7	4 9	4 10	4 9	4 9	4 2	3 10	4 2	4 5	4 1
"	0.25	"	0.10	MH 92067	—	—	0 7	2 6	3 5	4 6	4 11	5 4	5 14	6 3	7 3	6 3	6 8	6 11	6 4	5 6	5 13	6 6

## EXPERIMENT III. MILL HILL

It was decided that the second lot of seven heifers available at Mill Hill should be used for the simultaneous injection of a slowly acting and of a rapidly acting ester or the free substance. The dilaurate and the dipalmitate in association with the dipropionate or the free substance were used. The dosage of the former was kept constant at 0.25 g.; that of the latter was varied from 0.05 to 0.25 g. Six of the heifers were injected on 14 May 1943, the seventh, no. 23601, on 1 September 1943, on which date two of the first six were re-injected.

*Milk yield*

The dosages and the milk yields are shown in Table 8. It will be seen that the two heifers (MH 26 and MH 30) receiving free stilboestrol in addition to the slowly acting ester failed to give appreciable amounts of milk. This result suggests that a small dose of the free substance not merely fails to synergize with the slowly acting material, but actually inhibits the effect which might otherwise be produced by the slowly acting ester alone (see Exp. I). The results with the other heifers were definitely encouraging. 0.100 g. of the dipropionate with the dilaurate and with the dipalmitate produced a better response than 0.05 g. (Figs. 2, 3), while the two heifers receiving dilaurate each gave a better yield than the corresponding one receiving dipalmitate. Increase of the dose of dipropionate to 0.25 g. in the last heifer (MH 23601) receiving dilaurate as the basal treatment produced the best response of all. This heifer was averaging more than 12 lb. per day in the twelfth week after injection and the yield was still rising (Fig. 3). This result must be considered very satisfactory for a heifer of the size used. Full allowance must, of course, be made for individual variation in response and for other chance factors, but the three heifers receiving 0.25 g. dilaurate and increasing amounts of the dipropionate gave most consistent results (Fig. 3), from which it seems permissible to draw the conclusion that the simultaneous injection of 0.25 g. of the dilaurate and 0.25 g. of the dipropionate is a highly effective treatment for inducing lactation, probably of the same order of effectiveness as the implantation of tablets.

However, it should be recorded that a second treatment on these lines of heifers 26 and 30, which failed to respond after the first injection, did not result in appreciable lactation. It is known that it is more difficult to evoke a response from a mammary gland which has already been stimulated (cf. the dry cows used by Hammond & Day).

Of the five heifers that responded, two (MH 16535 and MH 92067) came into milk (over 1 lb. daily) in the fourth week after treatment, the other three only in the fifth, sixth and seventh weeks. It is difficult to connect this result with dosage, since the three in which lactation was delayed included the two receiving the lowest dose and the one receiving the highest dose of dipropionate. However, the greatest delay in producing any milk at all was seen in the heifer which received the large dose of dipropionate and which might, therefore, be expected to have undergone the greatest pre-secretory development of the mammary gland tissue and the longest postponement of the secretory phase. In general, the latent period before the appearance of milk was greater in the heifers injected with the esters than in the heifers implanted with tablets, recorded by our colleagues.

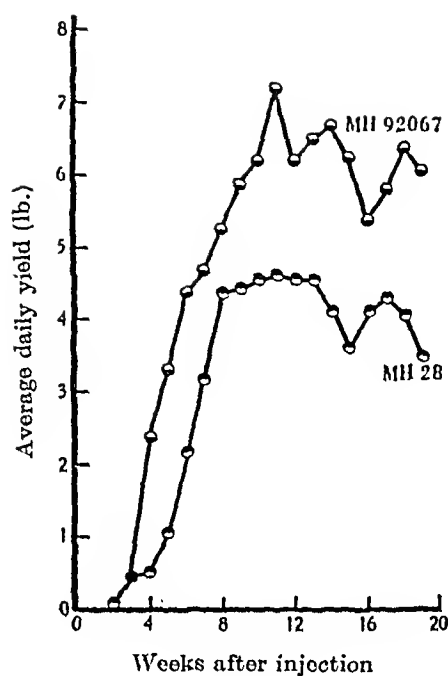


FIG. 2.

FIG. 2. Exp. III. Milk yields of heifers receiving simultaneous injections of diethylstilboestrol dipalmitate and dipropionate. MH 28, 0.25 g. of diethylstilboestrol dipalmitate; 0.05 g. of diethylstilboestrol dipropionate. MH 92067, 0.25 g. of diethylstilboestrol dipalmitate; 0.10 g. of diethylstilboestrol dipropionate.

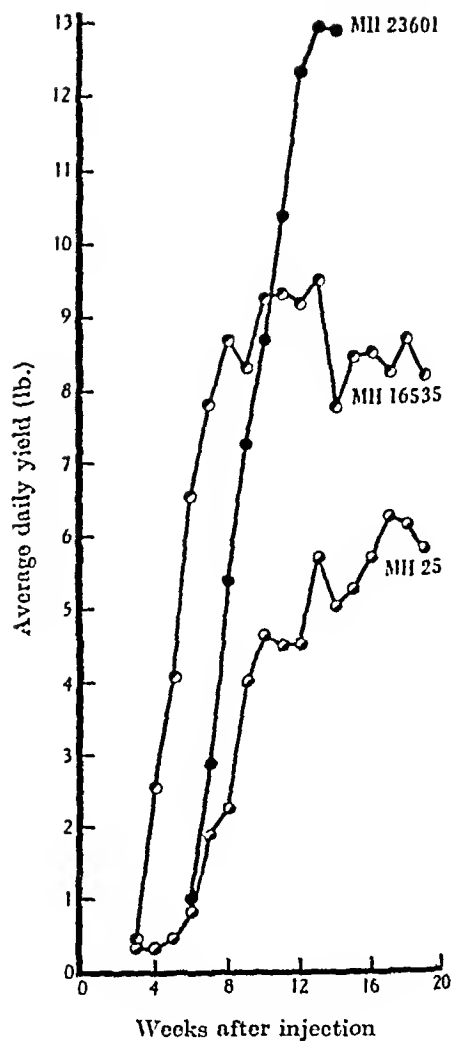


FIG. 3.

FIG. 3. Exp. III. Milk yields of heifers receiving simultaneous injections of diethylstilboestrol dilaurate and dipropionate. MH 25, 0.25 g. of diethylstilboestrol dilaurate; 0.05 g. of diethylstilboestrol dipropionate. MH 16535, 0.25 g. of diethylstilboestrol dilaurate; 0.10 g. of diethylstilboestrol dipropionate. MH 23601, 0.25 g. of diethylstilboestrol dilaurate; 0.25 g. of diethylstilboestrol dipropionate.

Table 9. *Experiment III. Milk composition*

No. of heifer		29. vi. 43	11. viii. 43	7. ix. 43	13. x. 43	26. x. 43
MH 30	Solids non-fat %	—	—	—	—	9.4
	Fat %	—	—	—	—	4.8
MH 25	Solids non-fat %	9.0	8.7	9.2	9.7	8.6
	Fat %	4.6	4.0	3.4	3.8	4.3
MH 16535	Solids non-fat %	9.2	8.6	8.6	8.7	9.2
	Fat %	5.2	4.0	4.9	5.3	4.4
MH 23601	Solids non-fat %	—	—	—	9.7	9.2
	Fat %	—	—	—	4.2	4.6
MH 28	Solids non-fat %	9.2	8.5	8.8	8.6	8.6
	Fat %	5.0	3.9	3.8	5.2	5.6
MH 92067	Solids non-fat %	9.4	8.5	8.9	8.9	8.9
	Fat %	5.1	4.4	5.5	—	—



Table 10. *Experiment III. Occurrence of oestrus and condition of the ovaries*

No. of heifer	Periods of oestrus	Condition of ovaries						
		11. v. 43	2. vi. 43	9. vii. 43	23. viii. 43	16. ix. 43	30. ix. 43	15. xi. 43
M11 30	31. vii. 43 to 1. viii. 43	R.O. Neg.	Neg.	R.O. f.	R.O. Neg.	Neg.	Neg.	R.O. 'Cherry' cyst
	31. viii. 43 to 2. ix. 43	L.O. c.l.		L.O. 'Cherry' cyst	L.O. c.l.			L.O. Neg.
	10. ix. 43 to 12. ix. 43							
	27. v. 43 to 29. v. 43	Neg.	Neg.	R.O. Neg.	R.O. Neg.	R.O. c.l.	R.O. c.l.	Neg.
M11 25	13. vii. 43 to 14. vii. 43			L.O. c.l.	L.O. s.c.l.	L.O. Neg.	L.O. Neg.	
	1. x. 43 to 3. x. 43							
	10. xi. 43 to 11. xi. 43							
	16. v. 43 to 11. v. 43	R.O. f.	Neg.	R.O. c.l.	Neg.	R.O. c.l.	Neg.	R.O. Neg.
M11 10535	27. v. 43 to 28. v. 43	L.O. Neg.		L.O. Neg.	L.O. Neg.	L.O. Neg.		L.O. c.l.
	28. vi. 43 to 29. vi. 43							
	16. vii. 43 to 16. vii. 43							
	1. viii. 43 to 2. viii. 43							
M11 23061	28. viii. 43 to 30. viii. 43							R.O. 'Cherry' cyst
	18. x. 43 to 19. x. 43							L.O. Neg.
	8. xi. 43 to 9. xi. 43	Neg.	Neg.	Neg.	R.O. c.l.	Neg.	Neg.	
	9. viii. 43 to 11. viii. 43				L.O. Neg.			
M11 20	29. viii. 43 to 30. viii. 43							
	16. ix. 43 to 11. ix. 43							
	21. ix. 43 irregularly in season every few days to 8. x. 43							
	19. v. 43 to 20. v. 43	R.O. c.l.	Neg.	R.O. f.	R.O. Neg.	Neg.	Neg.	R.O. 'Cherry' cyst (small)
M11 28	10. vi. 43 to 17. vi. 43	L.O. Neg.		L.O. Neg.	L.O. c.l.			L.O. Neg.
	23. viii. 43 to 23. viii. 43							
	26. x. 43 to 28. x. 43							
	15. xi. 43 to 16. xi. 43							
M11 92067	12. vii. 43 to 13. vii. 43	Neg.	Neg.	R.O. c.l.	Neg.	R.O. s.c.l.	Neg.	R.O. Neg.
	8. x. 43 to 9. x. 43			L.O. Neg.		L.O. Neg.		L.O. c.l.
	29. x. 43 to 30. x. 43							
	19. xi. 43 to 20. xi. 43							
M11 92067	19. v. 43 to 20. v. 43							
	21. vii. 43 to 22. vii. 43	Neg.	Neg.	R.O. Neg.	R.O. c.l.	R.O. c.l.	Neg.	Neg.
	8. ix. 43 to 9. ix. 43			L.O. f.	L.O. Neg.	L.O. s.c.l.		
	28. ix. 43 to 30. ix. 43							
M11 92067	23. x. 43 to 25. x. 43							
	13. xi. 43 to 14. xi. 43							

R.O. = right ovary; L.O. = left ovary; c.l. = corpus luteum; s.c.l. = small corpus luteum; Neg. = ovary (ies) quiescent; f. = follicle

*Milk composition*

The composition of the milk of the heifers in Exp. III sampled at intervals is shown in Table 9. No comment is necessary except to say that the fat content was above the legal minimum in all samples, and was definitely high in some. The solids non-fat content was normal to high.

*Oestrous cycle and condition of ovaries*

The data are given in Table 10. Three of the six heifers first injected had quiescent ovaries 3 days before treatment. Three weeks after treatment all were quiescent: 5 weeks later all had active ovaries. The two heifers reinjected on 1 September 1943, and the one injected for the first time on that date, all had inactive ovaries on 16 September 1943. The fertility of these animals has not yet been investigated.

## SUMMARY

1. Lactation can be induced in small virgin heifers by a single injection of diethylstilboestrol in ester form.
2. The simultaneous injection of a slowly acting and a rapidly acting ester seems to be the most effective method.
3. The best yield, a daily average at the peak period of 13 lb., was obtained from a heifer injected with 0.25 g. of dilaurate and 0.25 g. of dipropionate.
4. Allowing for the small size of the heifers, this combination of esters, given as a single injection, seems to induce lactation as effectively as does the implantation of tablets of diethylstilboestrol or hexoestrol.

Our best thanks are due to Dr W. S. Gordon, Director of the Field Station at Compton, for his ready co-operation in the second experiment, and to Prof. F. G. Young who was associated with us in the early stages of the work. We are greatly indebted to the following for practical assistance: Dr Ruth Deanesly, who carried out the oestrogen assays, Prof. M. F. Delafield in whose Department the milk analyses were made, Dr P. G. Marshall (British Drug Houses) who prepared the esters not ordinarily available, and Mr D. N. Spriggs, M.R.C.V.S., who carried out the ovarian examinations.

The Compton heifers were bought and maintained by the Agricultural Research Council; the Mill Hill heifers by the Medical Research Council.

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# EFFECTS OF SEX HORMONES ON THE BLOOD IN RATS

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(Received 24 January 1944)

It has been shown previously that the male sex hormones exert a stimulating effect on heart, liver, and kidney tissues, and on the general body growth of rats, while the effect of female sex hormones is predominantly depressing [Korenchevsky & Ross, 1940; Korenchevsky & Hall, 1941; Korenchevsky, Hall, Burbank & Cohen, 1941]. To see whether the haemopoietic tissues would also be susceptible to these stimulating or depressing influences the effects of gonadectomy, oestrus, and sex hormones on red cell and haemoglobin levels were studied and are described in this paper.

The clinical and experimental literature on the subject is largely contradictory, and a review of it is omitted at the Editors' request to economize space.

## METHODS

The effects of gonadectomy and of various combinations of hormones on the blood were studied in seventy-two male rats and forty-three females. As far as possible, litter-mates were equally distributed between different groups. The general arrangement of these experiments and the number of rats in each group can be seen from Tables 1 and 2. In addition, the effect of oestrus on blood was studied in thirty-eight female rats. Gonadectomy was performed at the age of 23–25 days, the rats being killed 100–150 days after the operation. In the experiments in which some rats received substances by injection, the remaining rats were injected with similar volumes (0.2 ml.) of pure arachis oil.

All the rats in the experiments summarized in the tables and fourteen rats in the 'oestrus' experiment were killed by bleeding from the abdominal aorta, the blood being collected for examination. From the remaining rats of the 'oestrus' experiment blood was collected from the tail.

The haemoglobin was estimated by Haldane's method, using the National Physical Laboratory standard. Red cell counts and haematocrit estimations were made with standardized apparatus.

The blood findings in adult rats of the same stock, bred and kept under the same conditions, showed comparatively little variation, and since any experimental changes were of the same order, similar groups have been pooled in the tables of results in order to save space. Each experiment was analysed statistically.

These analyses, using Fisher's *t*-test, were performed by Mrs B. Clapham and Mr J. L. Nicholson, to whom we are most grateful for this help. When assessing statistical significance, 's.s. < 1 : 100', for example, means that the observed difference could occur by chance less than once in 100 such experiments.

\* Dr Korenchevsky desires to express to the University of Oxford and to Prof. E. S. Goodrich his own and the Lister Institute's gratitude for the kind hospitality extended to him and his co-workers in the Department of Zoology and Comparative Anatomy.



Table 1. *Effects of castration and of hormones on the blood of male rats*

	Adult rats								
	Senile rats		Castrated rats treated with						
	Control	Injected with androgens	Control rats		Androgens	Oestradiol b.-b.	Androgens + oestradiol b.-b.	Androgens + oestradiol b.-b. + thyroid	Thyroid
			Normal	Castrated					
No. of rats in group	8	8	10	15	9	5	6	6	5
Red cells (millions per cu. mm.)	7.16	8.02	6.76	6.66	7.26	6.42	6.86	7.19	7.01
Haemoglobin (%)	86	106	95	91	100	88	91	99	93
Volume of packed cells (%)	40.1	45.4	39.5	39.7	43.5	38.7	40.9	43.7	41.6
Mean corpuscular volume (cu. $\mu$ )	56.2	57.0	58.6	59.5	60.5	60.4	60.0	60.6	59.7
Colour index	0.60	0.64	0.70	0.68	0.69	0.67	0.66	0.69	0.66
Abdominal fat (g.)	13.0	11.8	8.1	12.0	11.2	10.8	11.3	7.1	5.6
No. of rats examined in which fatty (F) or red (R) bone marrow was found	—	—	10 R 0 F	5 R 10 F	5 R 4 F	4 R 1 F	3 R 3 F	6 R 0 F	4 R 1 F

Table 2. *Effects of ovariectomy and of hormones on blood of female rats*

	Ovariectomized rats injected with			
	Control rats		Oestradiol b.-b.	
	Normal	Ovariectomized	Oestradiol b.-b.	Oestradiol b.-b. + progesterone
Number of rats in group	6	12	11	14
Red cells (millions per cu. mm.)	6.69	6.86	6.68	5.95
Haemoglobin (%)	94	93	90	82
Volume of packed cells (%)	39.7	40.8	40.2	35.5
Mean corpuscular volume (cu. $\mu$ )	60.3	59.6	60.1	61.8
Colour index	0.71	0.68	0.68	0.70
Abdominal fat (g.)	13.5	15.1	12.3	11.2
No. of rats examined in which red (R) or fatty (F) bone marrow was found	0 R 6 F	3 R 9 F	7 R 4 F	8 R 2 F

## RESULTS

*Experiment I. Normal senile male rats*

In this experiment (Table 1, cols. 1 and 2) the rats whose average age at death was about 2 years were injected once a week for 18 weeks with an androgenic mixture, each dose of 0.2 ml. containing 0.2 mg. each of testosterone propionate, testosterone dipropionate, and androsterone dissolved in arachis oil.

Injections of androgens increased the number of red cells by about 12 % (s.s. < 1:50), haemoglobin content by 19 % (s.s. < 1:100), volume of packed blood cells by 13 % (s.s. < 1:100), and the colour index by 7 % (s.s. < 1:35). The mean corpuscular volume was not changed.

*Experiments II and III (combined). Adult male rats*

The experiments (Table 1, cols. 3-9) were performed on ten intact rats serving as normal controls (col. 3), fifteen castrated controls (col. 4), and thirty-one castrated rats treated with various hormones. The rats were about 5 months old when killed.

In one experiment the same mixture of androgens was used as in Exp. I, but it was injected twice a week. In the other experiment 0.25 mg. of testosterone propionate alone was injected three times a week (col. 5, pooled results). 8  $\mu$ g. of oestradiol benzoate-butyrate (col. 6) were injected three times only during the first week of the experiment. Desiccated thyroid (Parke, Davis and Co.) was given every alternate or every day (according to the reaction of the rats) in a single dose of 65 mg. by pipette as a sweetened emulsion (col. 9). To the rats of the two remaining groups the same doses of androgens and oestradiol benzoate-butyrate (col. 7) or of all three hormones (col. 8) were given simultaneously. The experiment lasted 6 weeks.

*Castration* did not produce any significant effects on the blood values examined (compare cols. 3 and 4).

The effect of *androgens* on castrated rats (Table 1, col. 5) was similar to that on senile intact rats in Exp. I, except that there was no change in the colour index. On the average, as shown in Table 1, the number of red cells increased by about 9% (s.s. < 1 : 100) and the haemoglobin content and the packed cell volume by about 10% (s.s. < 1 : 100).

The oestradiol ester produced no effect (col. 6), while thyroid hormone (col. 9) caused a slight increase (statistically not significant) in red cells, haemoglobin and volume of packed cells.

However, when oestradiol benzoate-butyrate, even in doses which apparently have no effect on the blood, was injected simultaneously with androgens (col. 7) an antagonistic effect was produced by the oestrogen on the 'androgenic' changes. The administration of thyroid hormone simultaneously with androgens and oestradiol neutralized the antagonistic effect of the latter. Changes of about the same order (col. 8) and significance (s.s. < 1 : 50 for red cell count and volume of packed cells, < 1 : 100 for haemoglobin) were obtained as with androgens alone.

#### *Experiments IV and V (combined). Adult female rats*

Six intact normal controls (Table 2, col. 1), twelve ovariectomized controls (col. 2), and twenty-five ovariectomized injected rats were used in this experiment. The average age of the rats was about 5 months; the duration of the experiment 45 days. The same dose of oestradiol benzoate-butyrate was injected as in the previous experiments, alone (col. 3) or with progesterone (col. 4).

Starting 4 weeks after the administration of the first dose of oestradiol, 1-2 mg. of progesterone were injected into a special group (col. 4) of female rats daily for 17 days.

Comparison of cols. 1 and 2 shows that ovariectomy had no effect.

In the dose used, oestradiol benzoate-butyrate alone had no significant effect on the blood, but when injected simultaneously with progesterone the blood values were decreased (red cells, s.s. 1 : 40-60; haemoglobin, s.s. 1 : 20-35).

#### *Experiments VI-VIII. Effect of oestrus on blood of adult rats*

In Exp. VI, estimations were made of the number of red cells and of the haemoglobin content of blood taken from the tail of twelve rats in dioestrus and again from the same rats in oestrus. In Exp. VII similar examinations were made of blood from the tail of five rats in dioestrus and seven rats in oestrus (total twelve rats). The average age of all the above rats was 12 months. In Exp. VIII blood was examined

from the aorta of seven rats killed in dioestrus and seven rats killed in oestrus. These fourteen rats were about 4½ months old.

No effects of oestrus were observed in any of these experiments. The results were therefore pooled, the general averages obtained being strikingly close for each blood value. Thus, the averages of twenty-four blood examinations in rats in dioestrus were: red cells 7·31 millions, haemoglobin 102 %, and colour index 0·70; the averages of twenty-six blood examinations on rats in oestrus were exactly the same.

Blood taken from the tail gave slightly higher values for red cells and haemoglobin content than did that from the aorta. Comparison of the figures from the ten rats from which blood was taken from both tail and aorta showed that this difference was very small—only about 3 %.

#### *Effect of age and sex on blood of rats*

No significant differences were found between the blood values in male and female control rats (compare cols. 3 and 4 in Table 1 with cols. 1 and 2 in Table 2), and hence no effect of sex could be detected on the blood of adult rats. It must be emphasized, however, that the apparently stimulating effect of androgens and depressing action of female sex hormones might suggest an explanation of the higher blood values of human males as compared with females, starting from the period of adolescence. The absence of such a difference in rats might be caused by a different balance of the hormones concerned in this animal.

If in Table 1 cols. 1 and 3 are compared, it can be seen that in blood of old rats there is a lower haemoglobin content and a slightly increased number of red cells, which, though separately not significant, gives a significantly low (s.s. < 1 : 100) colour index. Because of the small number of rats examined, however, it is not possible to draw a definite conclusion that this hypochromia is typical of old age. Williamson & Ets [1926] also found a decreased haemoglobin content in the blood of rats with ageing, even from the age of 6 months.

#### *Bone-marrow changes*

In the experiments on rats made by Vollmer, Gordon & Charipper [1942] and by Steinglass, Gordon & Charipper [1941], the presence of bone marrow with a network of fat cells was considered to be a sign of depressed haemopoiesis (hypoplastic bone marrow); bone marrow without fat cells, on the contrary, indicated stimulated haemopoiesis (hyperplastic bone marrow). We made an attempt to judge haemopoiesis in our rats by the same method, investigating histologically the bone marrow from the middle of the femur. The results are given in the tables, and at first glance they suggested that both castration and sex hormones produced definite effects. It seemed possible, however, that the fat tissue of bone marrow would reflect the changes in general fat deposition. Therefore in Tables 1 and 2 are also included the amount of abdominal fat (always weighed in our experiments) which accurately reflects the general fat deposition in the body of the rat. Comparison of the changes in the fat of the bone marrow with those in the abdominal fat indicates that a close correlation exists between these values. Therefore, in our opinion, the variation in content of fat tissue in bone marrow cannot be used as a reliable diagnostic feature of haemopoietic activity.

## DISCUSSION

The results of our experiments do not permit any conclusions to be drawn about haemopoietic activity for which blood-volume studies would be essential. Definite effects of the hormones on blood, however, were clearly shown and the results obtained with androgens were similar to those of Vollmer *et al.* [1942] and Steinglass *et al.* [1941]. The discrepancy in the results of different authors on the effect of oestrogens on blood is most probably due to differences in dosage; while small doses have most important physiological activities, large doses are known to produce toxic effects. Also our experiments were performed on litter-mates of known age, while most previous workers used mixed rats usually of unknown age. The absence of any effect of gonadectomy on the blood picture in our experiments and those of some other workers may perhaps be explained by the fact that some post-castration changes develop slowly; it is possible that blood changes might be detected at a later period after gonadectomy. If the results of our experiments are confirmed on human beings, an explanation of the anaemias often observed in pregnancy [Sodeman, 1940] might be found in the combined effects of oestrogenic and progestational hormones.

With regard to the cause of human idiopathic hypochromic anaemias, it is noteworthy to record that in our senile rats the low colour index was raised by androgenic treatment.

## CONCLUSIONS

1. The number of red cells, haemoglobin content, colour index, volume of packed cells and mean corpuscular volume in the blood of adult rats were not affected by sex of the animals, gonadectomy performed 100–150 days before the end of the experiment, nor by oestrus.
2. In eight senile rats a mild hypochromia was observed.
3. Androgens increased the number of red cells, haemoglobin content, and volume of packed cells in normal senile, and castrated adult, male rats, the lowered colour index of the senile rats being raised.
4. Small doses of oestradiol benzoate-butyrate injected alone into male or female rats had no effect on the blood values examined.
5. A definite decrease in the number of red cells, haemoglobin content, and volume of packed cells occurred when progesterone and oestradiol benzoate-butyrate were injected together into ovariectomized rats.
6. The effect of androgens in castrated rats was neutralized when oestradiol benzoate-butyrate was injected simultaneously, but not when thyroid hormone was given in addition to the androgens and oestrogen.
7. The results are discussed with reference to sex differences in the human blood picture and to the cause of pregnancy anaemias of women.
8. The amount of fat tissue in bone marrow apparently reflects the changes in general fat deposition and not changes in haemopoiesis.
9. Our experiments, while showing definite effects of sex hormones on the blood, do not explain whether the results obtained were due to changes in blood volume or in haemopoiesis.

Grants from the Medical Research Council and the Lister Institute and the facilities extended to us by Dr A. H. T. Robb-Smith and Dr R. G. Macfarlane, at the Pathological Laboratory, Radcliffe Infirmary, Oxford, have enabled us to carry out this work, and to them we offer our thanks. We are also grateful to the Ella Sachs Plotz Foundation for a grant from which some technical expenses were met; to Mr B. Amos for his skilful technical help; to Ciba Ltd. and Organon Laboratories Ltd. for the generous supply of hormones; and to Parke, Davis and Co. for the desiccated thyroid.

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# THE INFLUENCE OF THE THYROID ON PREGNANCY AND PARTURITION IN THE RABBIT

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(Received 27 January 1944)

The ovarian changes in the rabbit following complete thyroidectomy have been reported in a previous communication [Chu, 1944]. It was pointed out that in the absence of the thyroid gland, hypertrophy of the follicular apparatus resulted from the increased production of follicle-stimulating hormone, but that the life span of the corpus luteum did not seem to be shortened although the pituitary luteinizing hormone was diminished. As a result of follicular hypertrophy, the oestrogen in the circulation would be increased. Since such an increase over a certain physiological level would disturb the course of pregnancy [Parkes, Dodds & Noble, 1938; Heckel & Allen, 1938; and others], an investigation into the relationship between the thyroid gland and gestation in the rabbit was undertaken.

## MATERIAL AND METHOD

The experimental animals were divided into four groups: (1) normal rabbits were rendered pregnant and were thyroidectomized at various times during gestation; (2) pregnancy was induced in thyroidectomized rabbits; (3) pregnancy was induced in thyroid-fed, thyroidectomized rabbits; and (4) thyroid feeding was started in thyroidectomized rabbits after successful mating.

All the animals were kept in separate cages and the course of gestation was carefully watched. They were fed on the same diet and were kept under identical laboratory conditions.

## RESULTS

### *The effects of thyroidectomy in pregnant rabbits*

Eleven rabbits were thyroidectomized between the 2nd and 29th day of gestation. The animals survived the operation very well and no disturbance of any kind was

Table 1. *The effect of thyroidectomy in pregnant rabbits*

Rabbit no.	Time of thyroidectomy during gestation days	Gestation period days	No. of foetuses	Condition of foetuses
R 174	8	Embryos resorbed	—	—
R 146	20	27	4	Dead
R 148	29	30	5	Living
R 149	27	31	4	Dead
R 150	9	23	3	Dead and underdeveloped
R 147	10	24	—	Foetuses eaten by the mother
R 203	18	30	2	Dead and underdeveloped
R 153	2	30	4	1 living and 3 dead
R 196	4	25	—	Foetuses eaten by the mother
R 197	4	27	2	Dead and underdeveloped
R 212	5	30	5	2 living and 3 dead; all underdeveloped

observed. The results of these experiments varied in individual cases. In the majority of the thyroidectomized rabbits, pregnancy was terminated by the delivery of dead foetuses which were usually underdeveloped. As is shown in Table 1, there were very few thyroidectomized rabbits which could carry the pregnancy to full term and deliver normal litters, though the number of foetuses was in every case within the normal range. It may be concluded that thyroidectomy in the pregnant rabbit interferes with the normal course of gestation, but exerts no effect on the number of foetuses.

### *Pregnancy in thyroidectomized rabbits*

Fourteen thyroidectomized rabbits were rendered pregnant 17–108 days after operation. Since the thyroidectomized animals do not ovulate after coitus and the rupture of follicles usually follows the injection of chorionic or pituitary gonadotrophin [Chu, 1944], pregnancy was induced by mating followed by a single injection of chorionic gonadotrophin. When these animals had become pregnant, they were kept under close observation during the whole period of gestation.

The results of these experiments were not quite similar to those of the first group. As regards the length of the gestation period, it appeared to be shortened in some cases as a result of abortion, while in others it was obviously lengthened owing to the retention of foetuses. In some cases the embryos were completely resorbed at the mid-term of pregnancy. The act of parturition in this group of animals may last a few days and the foetuses may be retained in the uterus as long as one week. Very few of the foetuses were alive at birth, and these died shortly after delivery. The results of these experiments are summarized in Table 2.

Table 2. *Pregnancy and parturition in thyroidectomized rabbits*

Rabbit no.	Time of coitus after thyroidectomy days	Gestation period days	No. of foetuses	Condition of foetuses	Remarks
R 45	95	28	1	Dead	A few foetuses retained
R 47	108	30	1		
		32	2	Dead	One foetus not completely differentiated
		33	3		
		34	1		
R 48	91	32	2	Dead	A few foetuses retained
R 53	91	33	2	1 living and	—
		34	1	2 dead	
R 67	43	34	3	Dead	—
R 1	66	32	3	2 living and 1 dead	Thyroid remnants discovered
R 2	67	34	—	Dead	Some foetuses eaten
R 39	37	—	—	—	Embryos resorbed
R 81	40	—	—	—	"
R 105	17	27	—	—	Foetuses eaten
R 106	17	26	4	Dead	—
R 172	56	26	—	Dead	Some foetuses eaten
R 146	27	32	6	Half dead	All foetuses underdeveloped
R 174	63	—	—	—	Embryos resorbed

The maternal behaviour, such as plucking of the fur and gathering of the hay for making the nest, was not observed in these animals. The newly born foetuses, if alive, were usually deserted by the mother so that no living young could possibly be

brought up. Lactation seemed to be normal; as drops of milk could be expressed from the teats even several days after delivery.

Superfecundation was successfully induced in the thyroidectomized rabbits. As reported in the previous paper [Chu, 1944], the growth of the ovarian follicles is much enhanced by the removal of the thyroid gland. When such thyroidectomized animals were given a single injection of chorionic gonadotrophin, superovulation invariably resulted. If the ruptured ova were mature and fertilizable, it should be possible to produce superfecundation in such animals. Based on this assumption, we examined the exact number of embryos in four of the thyroidectomized rabbits at the mid-term by laparotomy. They contained 15, 16, 18, and 22 embryos. In our breed of rabbit the normal litter size is about 5, varying individually from 2 to 8 embryos. Thus, in the thyroidectomized rabbits, the litter size *in utero* may be several times greater than the normal.

Despite the large number of embryos the number of foetuses born at term was within the normal range since most of the foetuses are resorbed. In several cases pregnancy was terminated prematurely as a result of abortion or complete resorption of the embryos. It is clear, therefore, that superfecundation may be produced in thyroidectomized rabbits, but it cannot be maintained to full term without some form of treatment.

*Pregnancy in thyroid-fed thyroidectomized rabbits*

Four thyroidectomized rabbits were given desiccated thyroid at various intervals after operation. Each animal received 30 mg. of the drug per kilogram of body weight on alternate days. The body weights were recorded every other day and no ill effects were observed. They mated at various times during the period of thyroid feeding and pregnancy invariably resulted (Table 3). At parturition, half of the animals delivered normal litters of young, while the other half expelled at term dead foetuses which were otherwise quite normal. The length of the gestation period varied from 30 to 32 days. These results suggest that reproduction in the thyroidectomized rabbits is fairly normal when adequate doses of desiccated thyroid are given.

Table 3. *Pregnancy and parturition in thyroid-fed thyroidectomized rabbits*

Rabbit no.	Time of thyroid feeding after thyroidectomy days	Time of mating after thyroid feeding days	Amount of thyroid given mg./kg./2 days	Gestation period days	No. of young	Condition of foetuses
R 195	48	14	30	31	3	Dead; normal size
R 205	28	14	30	32	5	Living; normal size
R 207	25	14	30	30	4	Dead; normal size
R 208	79	67	30	31	4	Living; normal size

*Maintenance of superfecundation by thyroid therapy*

The failure of the thyroidectomized rabbit to maintain its supernumerary embryos suggests that thyroid hormone may be essential for the maintenance of pregnancy. The restoration of this hormone to a physiological level might improve the maintenance of superfecundation. Six thyroidectomized rabbits were rendered pregnant by coitus followed by injection of pituitary gonadotrophin and divided into two groups. Three animals were given 30 mg. of desiccated thyroid per kilogram of body



weight every other day, and no treatment of any kind was given to the other group of three animals. The results showed that embryo resorption and abortion occurred in most of the non-treated animals (Table 4). On the other hand, one of the thyroid-fed

Table 4. *The effect of thyroid feeding on the maintenance of superfecundation in thyroidectomized rabbits*

Rabbit no.	Occurrence of gestation after thyroidectomy days	Amount of thyroid given mg./kg./2 days	Gestation period days	No. of foetuses	Pregnancy condition	Remarks
R 172	55	0	—	15	Abortion	A few dead foetuses delivered
R 174	60	0	—	18	Abortion	" "
R 146	27	0	30	16	Full term	" "
R 173	131	30	28	—	Premature labour	Many foetuses eaten
R 153	93	30	—	12	—	Died on 26th day of gestation; foetuses normal
R 197	90	30	—	13	—	" "

rabbits delivered a number of dead foetuses on the 28th day of gestation. The exact number of the young was not known, as several had been eaten by the mother at birth. The remaining two rabbits died suddenly on the 26th day of pregnancy. It was found at death that the abdominal cavity of these animals was fully loaded with embryos, and in one case the abdominal wall had burst at the substernal region. Dissection revealed 13 well-developed foetuses in one case and 12 in the other. These results indicate that thyroid feeding had prevented the resorption of the embryos to a certain extent. There is no evidence as to whether an increase in thyroid dosage would completely prevent resorption and maintain the gestation to full term. At present it appears certain that the thyroid gland is particularly important for the vitality and growth of the embryos; resorption or abortion occurs usually after the death of the foetuses.

#### DISCUSSION

Krichesky [1939] reported that thyroid removal in the rabbit from 1½ hr. to 12 days after mating does not alter the gestation period or the histology of the ovary. He claimed that thyroidectomized rabbits were mated again after their first delivery and successfully terminated a second pregnancy within the limits of a normal process of parturition. According to the results of this author, the thyroid gland has nothing to do with gestation and parturition in the rabbit. This conclusion is obviously at variance with the results of the present study.

The importance of the thyroid hormone lies in its ability to maintain the vitality and growth of the embryos during gestation. If the death of the embryos ensues at an early stage of pregnancy, resorption takes place. On the other hand, if the embryos die at a later stage of pregnancy, abortion occurs. Both of these phenomena were found in the thyroidectomized rabbits. It is reasonable to conclude that the thyroid hormone is of paramount importance in maintaining the vitality of the embryos.

Cases of thyroidectomized animals which had been able to carry the pregnancy to full term were not lacking. However, on delivery, the young were mostly dead

and underdeveloped. This indicates that the growth of the embryos had been retarded by thyroidectomy. Inasmuch as the external organs of the foetuses were complete and adequately developed, the extirpation of this gland does not interfere with differentiation and organogenesis of the embryos.

When pregnancy was induced in rabbits thyroidectomized for a long time, the gestation period may be considerably lengthened and the act of parturition may become intermittent. Preliminary tests on the contractility of the uterine muscle from thyroidectomized rabbits have not revealed the cause. The underlying mechanism may be found in the excess oestrogen which is supposed to be created in the thyroidectomized animals. Prolongation of pregnancy in the rabbit by the injection of oestrogenic hormone was reported by Heckel & Allen [1938]. This is due to the sustaining effect of the oestrogen on the secretory activity of the corpus luteum. The injection of oestrone in pseudopregnant rabbits postpones the regression of the corpora lutea [Chu & Lee, 1942], and the life span of this organ is lengthened in the thyroidectomized rabbit [Chu, 1944]. These facts may be considered as evidence for the over-production of oestrogen under hypothyroid conditions. The prolongation of pregnancy in thyroidectomized rabbits may thus be explained.

Experimental superovulation and superfecundation have been reported by Pincus [1940] and Evans & Simpson [1940] respectively. Their methods mainly involve the injection of gonadotrophic preparations of pituitary or placental origin, thereby accelerating the growth of the graafian follicles. No other method has yet been reported which can produce a similar stimulating effect on the ovarian follicles. Both superovulation and superfecundation have been successfully produced in the thyroidectomized rabbits, which strongly supports our hypothesis that the follicle-stimulating hormone of the pituitary is greatly increased in the absence of the thyroid [Chu, 1944].

The maintenance of superfecundation is rather a complicated problem. The supply of thyroid hormone is certainly one of the factors involved. As we have pointed out that the thyroid is essential for the vitality and growth of the embryos and that the administration of desiccated thyroid to the superfecund animals prevents the resorption of the embryos, there must be some other factors which are responsible for the abortive termination of superfecundation. Among these factors the nutritional requirements of the embryos and the maximum capacity of the uterus are the most outstanding. If we could supply the embryos with adequate nutrients and the number of embryos does not exceed the limit of uterine capacity, large litters might be obtained.

#### SUMMARY

Thyroidectomy in the rabbit at an early stage of pregnancy caused resorption and abortion of the embryos, while the operation performed at a late stage of pregnancy resulted in the delivery of still-born young. When pregnancy was induced in thyroidectomized rabbits, the results have been either resorption of the embryos, or abortion, or prolongation of gestation owing to retention of the foetuses. The new-born foetuses of the thyroidectomized animals were usually dead. Thyroidectomized rabbits fed with desiccated thyroid following the operation gave normal viable litters in two out of four cases.

Superfecundation had been successfully induced in thyroidectomized rabbits. The large number of embryos thus produced was usually resorbed. Embryo resorption could be prevented by desiccated thyroid therapy started immediately after mating.

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# THE ROLE OF THYROID GLAND AND OESTROGEN IN THE REGULATION OF GONADOTROPHIC ACTIVITY OF THE ANTERIOR PITUITARY\*

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(Received 27 January 1944)

It has been generally accepted that the secretion of gonadotrophic substances by the pituitary may be inhibited by sex hormones. This specific action of sex hormone on the pituitary is not supposed to be related to the functional state of other glands. Recently, Chu & Lee [1942] called attention to the fact that complete thyroidectomy in the rabbit causes hypertrophy of follicles, but prevents ovulation occurring after coitus. They also produced evidence to show that the failure of ovulation in the thyroidectomized rabbits is not due to the loss of sensitivity of the ovary nor is it due to inhibition of the reflex initiated by coitus. They believe that the formation of the ovulating hormone may be prevented by extirpation of the thyroid. On the other hand, the follicular hypertrophy is an indication of increased secretion of follicle-stimulating hormone by the pituitary. If we assume that the pituitary of thyroidectomized rabbits contains very little ovulating hormone but large amounts of follicle-stimulating hormone, then the injection of extracts of such pituitaries into the oestrous rabbit should cause no ovulation but should increase the size of the follicles. This is found to be the case [Chu, 1943]. The evidence thus far accumulated indicates that the thyroids as well as the gonads may be concerned in the regulation of the gonadotrophic function of the anterior pituitary.

A further result of follicular hypertrophy, through the increased production of oestrogen, should be the inhibition of follicle-stimulating secretion so that the follicular hypertrophy should eventually disappear in rabbits thyroidectomized for a long time. In our experience this has, however, not been the case. Unless we assume that the growth of follicles is not necessarily accompanied by an increased secretion of oestrogen, we have every reason to assume that oestrogen cannot exert its action on the pituitary in the absence of the thyroid.

We have therefore carried out the following experiments with the object of determining the exact role played by the thyroid gland and oestrogen in regulating the gonadotrophic activity of the pituitary.

## MATERIAL AND METHODS

Female rabbits of the local breed were exclusively used for the experiments. All the animals were kept in wire cages under the same conditions. The experiments were divided into five parts: (1) stilboestrol was injected subcutaneously into normal and thyroidectomized rabbits; (2) thyroidectomized rabbits were fed on desiccated thyroid in various doses; (3) thyroidectomized animals were treated simultaneously

\* A preliminary note has been published: *Proc. Chinese Physiol. Soc. Chengtu Branch*, 2, 6 (1943).

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with oestrogen and various doses of desiccated thyroid; (4) normal rabbits were injected subcutaneously with small doses of oestrogen with or without feeding of desiccated thyroid; and (5) normal rabbits were fed moderate and high doses of desiccated thyroid.

The crystalline oestrogens, stilboestrol and oestrone, were dissolved in sesame oil at a concentration of 2 mg. per ml. Desiccated thyroid (Lilly) was administered on the basis of body weight. The animals were weighed before feeding.

At the end of an experiment, each animal was injected intravenously with 30 i.u. of International Standard chorionic gonadotrophin or 5 mg. of ox pituitary extract in order to determine the ovulatory capacity of the ovary. It was killed 48 hours later and organs such as the ovary, oviduct, adrenal and thyroid glands were removed and fixed in Bouin's fluid for histological study. The anterior pituitary was finely macerated in saline, filtered and injected intravenously into oestrous or thyroidectomized rabbits for assay of its gonadotrophic potency. The use of thyroidectomized animals as test material was mainly due to their high sensitivity in response to the ovulating hormone of the pituitary [Chu, 1944].

Other experimental details will be described under the following separate headings.

## RESULTS

### *The effect of oestrogen injections on the pituitary of normal and thyroidectomized rabbits*

Five normal and ten thyroidectomized rabbits were treated with oestrogen. All the animals were given daily subcutaneous injections of 1 mg. of stilboestrol except two thyroidectomized animals which were given the same amount of oestrone. The injection period varied from 20 to 40 days for the thyroidectomized group and 20 to 35 days for the normal group. The body weights of the experimental animals varied considerably, the average being about 1.6 kg. All the normal females were sexually active and vigorous, otherwise they could not stand the injections of oestrogen for the desired length of time. This is in contrast to the thyroidectomized animals which tolerated the injection of oestrogen well.

The results obtained are presented in Table 1. In the intact animals, the ovary appeared to be normal or slightly depressed by the oestrogen after twenty injections. Ovulation occurred in all cases following the injection of chorionic gonadotrophin. When the injection of oestrogen was continued for 30 days, the number of follicles was reduced to a minimum and a high degree of ovarian inhibition was obtained. No ovulation was induced by the injection of chorionic gonadotrophin. If the animals received continuous injections for 35 days, follicles of different sizes were entirely absent and the ovary appeared to be smooth. The gonadotrophic potency of their pituitaries was assayed on oestrous rabbits. It was revealed that injection of such a pituitary extract neither caused rupture nor growth of follicles. This may indicate the absence of ovulating and follicle-stimulating hormones from the pituitary.

In thyroidectomized rabbits on the other hand, oestrogen injections were entirely ineffective in depressing pituitary activity. Although these animals received the treatment for a longer period than the normals, their ovarian activity was not much affected. The occurrence of ovulation in response to the injection of chorionic gonadotrophin and the presence of numerous follicles of various sizes strongly support this

Table 1. *Ovarian response to the injection of chorionic gonadotrophin in normal and thyroidectomized rabbits treated with stilboestrol (1 mg. daily)*

Rabbit no.	Injection period days	No. of ruptured follicles induced	Condition of ovary
(a) Thyroidectomized rabbits			
R 77	20	10	Several haemorrhagic and several large follicles
R 71	20	5	Numerous follicles of various sizes
R 97	20	1	Several large follicles
R 133	30	3	A number of large follicles
R 118	30	0	Considerable number of follicles
R 140	35	3	A number of large follicles
R 141	35	3	" "
R 173	40	3	" "
*R 161	40	1	Many follicles of various sizes
*R 8	40	1	" "
(b) Intact rabbits			
R 87	20	1	Normal appearance
R 01	20	2	Ovary contained few follicles
R 190	30	0	Very few follicles
R 88	35	0	No follicles
R 02	35	0	"

\* Treated with oestrone.

conclusion. However, as judged by the number of intact and ruptured follicles, a certain degree of inhibition may have occurred. Since the occurrence of accessory thyroid is quite common in various animals [Woelfler, 1883], this minor degree of inhibition might be attributed to the presence of some otherwise inactive accessory thyroid.

Pituitary assays had been made mostly on thyroidectomized rabbits. Such animals are not suitable for detecting the follicle-stimulating activity because their ovaries already contain an unusual number of large follicles. Ovulation tests were invariably negative. It is quite conceivable that this absence of ovulating hormone from the pituitary is characteristic of the thyroidectomized rabbit.

#### *The effect of thyroid feeding on the pituitary of thyroidectomized rabbits*

It was noted in a previous communication [Chu, 1944] that when desiccated thyroid is fed to thyroidectomized rabbits, the overgrowth of follicles is no longer evident and the content of ovulating hormone is restored to a normal level. This suggests that an increased amount of thyroid hormone decreases the content of follicle-stimulating hormone and increases the content of ovulating hormone. In so far as the follicle-stimulating hormone is concerned, the action of thyroid hormone is just the same as that of oestrogen in intact animals. As the oestrogen is not effective in suppressing the pituitary in the absence of the thyroid, it may be thought that the sex hormone may exert its effect by stimulating the thyroid which in turn affects the anterior pituitary. If this hypothesis is valid, the administration of small doses of desiccated thyroid to thyroidectomized rabbits would only prevent the ovary from an overgrowth of follicles, while large doses would suppress its activity by way of diminishing the follicle-stimulating content of the pituitary. The results of these experiments are shown in Table 2.

Table 2. *Ovarian response to the injection of ovulating substance in thyroid-fed thyroidectomized rabbits*

Rabbit no.	Dosage of thyroid mg./kg./2 days	Period of thyroid feeding days	Ovulation induced by	No. of ruptured follicles	Condition of ovary
R 217	30	40	30 i.u. PU*	3	Several small and medium-sized follicles
R 218	30	40	30 i.u. PU	3	" "
R 219	90	42	5 mg. ox p.	1	Only a few small follicles
R 220	90	40	5 mg. ox p.	1	Only a few large follicles present

\* PU=chorionic gonadotrophin; ox p.=ox pituitary gonadotrophin.

Among the four experimental animals, two were fed 30 mg. of desiccated thyroid per kg. body weight every other day for 40 days, and the other two received 90 mg. doses for the same period. At the end of the feeding period, they were given an intravenous injection of either chorionic, or ox pituitary, gonadotrophin in order to test the ovulation response and were killed 48 hr. later. Their ovaries were carefully examined and the gonadotrophic potency of their pituitaries was assayed.

The results showed that in those animals receiving small doses of thyroid, the general appearance of the ovary was not characteristic of hypothyroidism but similar to that in the normal rabbit. Three ruptured follicles were present in each case. Their pituitary extracts caused positive ovulation responses in oestrous rabbits. On the other hand, the ovaries of the two animals which received higher doses of desiccated thyroid showed an appreciable degree of functional inhibition, although some follicles of various sizes were still present and one ruptured follicle was observed in each case. Their pituitary extracts elicited one positive and one negative ovulation response in thyroidectomized rabbits. The results tell us clearly that, when the level of thyroid hormone is raised, the follicle-stimulating capacity of the pituitary is diminished and that of the ovulating hormone content increased (the latter being absent in the pituitary of thyroidectomized rabbits).

*The effect of simultaneous administration of oestrogen and desiccated thyroid on the pituitary of thyroidectomized rabbits*

We have shown clearly that the degree of thyroid activity is a determining factor in the fluctuation of pituitary gonadotrophic hormones. If the sex hormone acts upon the pituitary by stimulating the thyroid gland, the administration of various doses of desiccated thyroid together with a fixed amount of oestrogen to the thyroidectomized animals should not produce a further degree of ovarian atrophy than that caused by desiccated thyroid alone.

Four thyroidectomized rabbits were given injections of 1 mg. of oestrone per day for a period varying from 36 to 40 days. They were fed simultaneously 30 mg. of desiccated thyroid per kg. body weight on alternate days. Another two thyroidectomized rabbits were treated in the same way except that the dosage of thyroid was doubled. At the end of the experimental period, they received an intravenous injection of either chorionic, or ox pituitary, gonadotrophin as usual, and their pituitaries were assayed on oestrous or thyroidectomized rabbits. The results obtained are presented in Table 3.

Table 3. *Ovarian response to the injection of ovulating substance in thyroidectomized rabbits treated with oestrone (1 mg. daily) and thyroid*

Rabbit no.	Dosage of thyroid mg./kg./2 days	Experimental period days	Ovulation induced by	No. of ruptured follicles	Condition of ovary
R 26	30	40	30 i.u. PU*	1	Considerable number of follicles
R 11	30	40	30 i.u. PU	2	" "
R 12	30	36	5 mg. ox p.	5	" "
R VI	30	36	5 mg. ox p.	0	" "
R 9	60	35	30 i.u. PU	0	Very few visible follicles
R 205	60	40	30 i.u. PU	0	" "

\* PU=chorionic gonadotrophin; ox p.=ox pituitary gonadotrophin.

These results are comparable with those presented in Table 2. The addition of oestrogen in these cases did not alter the ovarian activity to a more profound degree than did the feeding of desiccated thyroid alone. As this amount of oestrogen would invariably inactivate the pituitary of normal rabbits, but only when normal amounts of thyroid hormone are present, it may be inferred that the thyroid intervenes in the process of interaction between the gonad and the pituitary.

The negative results of the pituitary assays indicate that the ovulating hormone can be directly inhibited by oestrogen injections.

*The effect of administration of small doses of oestrogen together with or without desiccated thyroid on the pituitary of normal rabbits*

The results of the foregoing experiments clearly demonstrate that oestrogen acts upon the pituitary indirectly through the thyroid. But the possibility that the thyroid hormone may potentiate the action of oestrogen is by no means excluded. It may be assumed that oestrogen acts upon the pituitary in such a way that its effect can only be fully expressed by the presence of thyroid hormone. In order to test this possibility the following experiments have been carried out.

Eight normal oestrous rabbits were divided into two groups. Of the first group of four animals, two received 0.25 mg. of oestrone per diem and the others received 0.5 mg. The administration of oestrogen to the second group of animals was exactly the same except that simultaneously they received 30 mg. of desiccated thyroid per kg. body weight on alternate days. The experiment lasted 40 days. A single injection of ox pituitary extract was given intravenously at the conclusion of experiment. The animals were autopsied 48 hr. later and an assay of the gonadotrophic potency of the pituitary was carried out.

The results of the experiments are summarized in Table 4. In the non-thyroid-fed group, oestrone at the dosage employed was not quite effective in suppressing the ovarian activity as indicated by the presence of ovulation in half of the treated animals and the presence of follicles of various sizes in all cases. On the other hand, the action of oestrogen was definitely re-enforced by the addition of desiccated thyroid. Ovulation was found in none of the four treated animals and the number of intact follicles was greatly reduced in each case. The results seem to support the view that the action of oestrogen may be potentiated by thyroid hormone. The significance of these findings will be discussed in some detail.



Table 4. *Ovarian response to the injection of ox pituitary extract in normal rabbits treated with small doses of oestrous with or without desiccated thyroid for 40 days*

Rabbit no.	Dosage of oestrous mg./day	Dosage of thyroid mg./kg./2 days	No. of ruptured follicles induced	Condition of ovary
R46	0.25	0	0	Considerable number of small and medium-sized follicles
R47	0.25	0	2	" " "
R50	0.50	0	0	" " "
R53	0.50	0	1	Several medium-sized follicles
R57	0.25	30	0	Ovarian surface smooth with a few small and large follicles
R59	0.25	30	0	Ovarian surface smooth with a few small follicles
R54	0.50	30	0	" " "
R56	0.50	30	0	Ovary almost deprived of follicles "

The results of pituitary assays showed invariably that the ovulating hormone was depressed by injections of the oestrogen either with or without the addition of desiccated thyroid.

*The effect of thyroid feeding on the pituitary of normal rabbits*

We have produced evidence to show that the depressant effect of oestrogen on the pituitary is closely related to the high concentration of thyroid hormone. Oestrogen can be rendered ineffective by the removal of thyroid gland and cannot exert any action on the pituitary when the concentration of thyroid hormone is limited. On this basis it should be possible to suppress pituitary function simply by raising the concentration of thyroid hormone in intact rabbits.

Seven normal oestrous rabbits were given 30 mg. of desiccated thyroid per kg. body weight every other day for a period of 35-40 days. Another two rabbits received much higher doses of this drug (90 mg.). A single dose of chorionic, or ox pituitary, gonadotrophin was as a rule injected intravenously at the end of the feeding period and the pituitary was assayed for its gonadotrophic potency.

Table 5. *Ovarian response to the injection of ovulating substance in normal rabbits treated with desiccated thyroid*

Rabbit no.	Dosage of thyroid mg./kg./2 days	Feeding period days	Ovulation induced by	No. of ruptured follicles	Condition of ovary
R31	30	40	30 i.u. PU*	6	A few large follicles and several small ones
R32	30	40	30 i.u. PU	1	" " "
R33	30	40	30 i.u. PU	6	" " "
R45	30	35	5 mg. ox p.	2	Moderate number of small and medium-sized follicles
R35	30	35	5 mg. ox p.	5	Some large and medium-sized follicles
R43	30	35	5 mg. ox p.	1	A few large and many small and medium-sized follicles
R44	30	35	5 mg. ox p.	1	Moderate number of small and medium-sized follicles
R221	90	19	—	—	Diagnosis of hyperthyroidism; ovarian surface was smooth with some small follicles
R226	60-90	40	5 mg. ox p.	2	Ovarian surface smooth with a few small and medium-sized follicles

\* PU=chorionic gonadotrophin; ox p.=ox pituitary gonadotrophin.

The results showed that in those animals receiving small doses of thyroid, ovulation was invariably induced by the injection of ovulating substance and the number of follicles was within the normal range (Table 5). Their pituitaries were quite effective in causing ovulation in oestrous or thyroidectomized rabbits. It is probable that the follicle-stimulating capacity of the pituitary was not much reduced by the small doses of desiccated thyroid. However, partial inhibition of the ovary was observed in those animals receiving high doses of thyroid. These two animals were at first fed 90 mg. of the drug per kg. body weight. They gradually became emaciated and one succumbed after 19 days of feeding. The dosage in the other rabbit was then reduced to 60 mg. for the rest of the experiment. The surface of the ovary was smooth with only a few small follicles in the rabbit that died of hyperthyroidism. The rabbit that survived the experiment had two ruptured follicles caused by the injection of ox pituitary extract. The surface of the ovary looked smooth with a few small and medium-sized follicles. The pituitary assay was negative.

#### DISCUSSION

The rhythmic activity of the sex gland is reflected in periodic changes of the pituitary. Grumbrecht & Loeser [1938] claimed, however, that the secretion of pituitary gonadotrophic hormones occurs at a constant rate but that the responsiveness of the ovary fluctuates in a rhythmic manner which is largely conditioned by the thyroid hormone. They are of the opinion that the oestrogen which is liberated by the ovary acts indirectly upon the thyroid by way of the uterus; in the uterus oestrogen either causes the liberation of, or is converted into, a substance having thyrotrophic properties. The ovary may thus be sensitized by thyroxine to the action of gonadotrophic hormones.

We agree with Grumbrecht & Loeser that oestrogen may activate the thyroid in some way, but there is no evidence to show that the action is direct, nor does the fact that extra-uterine administration is ineffective in the hands of the German authors suggest to us that the uterus is an intermediary in the action. We think that in all probability, the oestrogen may stimulate the pituitary to liberate thyrotrophic hormone which in turn activates the thyroid.

As has been reported in a previous communication [Chu, 1944] the thyroid acts upon the ovary by way of the pituitary. This is contrary to the hypothesis put forward by Grumbrecht & Loeser. Since the withdrawal of thyroid hormone by thyroidectomy brings about follicular hypertrophy and abolishes the mechanism of ovulation, the thyroid gland may thus affect the pituitary by suppressing the secretion of follicle-stimulating hormone and stimulating the production of luteinizing (or ovulating) hormone.

We have not as yet direct evidence of the hyperfunctional state of the thyroid under conditions of excess oestrogen but our results clearly suggest that this is so. In the first place, oestrogen when injected into long-thyroidectomized, or into recently-thyroidectomized, rabbits did not correct or prevent the follicular hypertrophy in the ovaries. Secondly, thyroidectomized rabbits when given physiological doses of desiccated thyroid did not respond to the injections of oestrogen by an atrophy of the ovary, but did so when large doses of the drug were administered. The same results can be obtained by feeding desiccated thyroid alone to thyroid-

ectomized rabbits. These facts all point to the possibility that the inhibitory action of oestrogen on the pituitary is caused by the stimulation of the thyroid. This assumption is strengthened by the finding that a considerable degree of ovarian inhibition has been successfully achieved by administering large amounts of desiccated thyroid to intact rabbits. The fact that small doses of desiccated thyroid were ineffective in this respect can be explained on the assumption that the animal's own gland may be suppressed by the introduction of extra thyroid [Schmidt & Schmidt, 1938]. The resultant of such physiological readjustment would make the total concentration of this hormone not much above the normal level.

Laqueur & Enge [1941] hold the view that the oestrogenic hormones depress the gonadotrophic apparatus more effectively in the presence of a hyperplastic thyroid. This would mean that the thyroid hormone may potentiate the action of oestrogen. Our experiments on normal rabbits treated with small doses of oestrogenic hormone with or without desiccated thyroid have yielded equivocal results. The fact that the effect of oestrogen was increased by the addition of thyroid can be taken as evidence to support the potentiation hypothesis. But small amounts of oestrogen may have activated the thyroid to a certain degree and the addition of some extra thyroid would make the total concentration of this hormone up to a level that may depress the pituitary effectively.

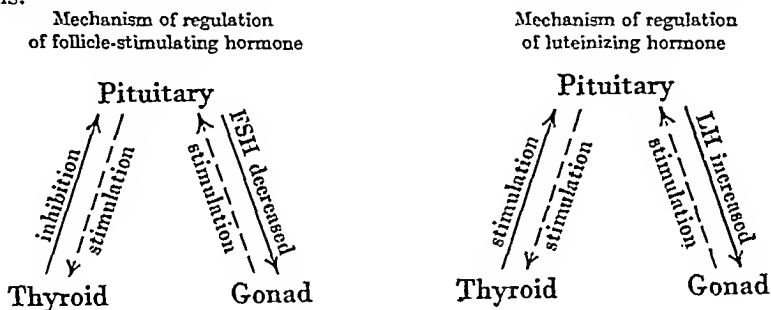
The two pituitary gonadotrophic hormones do not react in the same way to the changes of thyroid activity. From the body of evidence we may postulate that the production of both is under the regulation of the thyroid gland. When thyroid activity is depressed, the content of follicle-stimulating hormone in the pituitary is increased and the luteinizing hormone decreased. The reverse occurs on increasing thyroid activity: this is based on the observation that administration of desiccated thyroid to thyroidectomized rabbits causes a reduction in the number of follicles and the restoration of the ovulation response to coitus. Furthermore, the pituitary extract from thyroid-fed normal rabbits causes a higher percentage of ovulation in oestrous rabbits than does the pituitary extract from non-treated normal animals [Chu, 1943].

We know at present that an excess of oestrogen may inhibit elaboration of luteinizing hormone from the pituitary. But the possibility that a decrease in oestrogen concentration would result in an increased production of luteinizing hormone is still a matter of speculation. Since our knowledge as to the relationship between oestrogen and the pituitary is rather meagre and confused, we hesitate to accept the view that oestrogen is greatly concerned in the direct regulation of the gonadotrophic activity of the anterior pituitary.

Van Horn [1933] and Weichert & Boyd [1934] have shown that when large doses of desiccated thyroid are administered to oestrous rats the animals remain in a dioestrous condition for a long period. Two possible explanations have been suggested by the latter authors: either the thyroid stimulates the pituitary to elaborate a hormone which brings about the formation and persistence of corpora lutea; or the high metabolism may lower the oestrogen concentration to such a degree that the activity of the corpus luteum may be uninhibited. These explanations are apparently inadequate, and in the light of the present study the effect can be explained as follows. Since hyperthyroidism inhibits the production of follicle-stimulating hormone and

consequently the growth of follicles, dioestrus results from an oestrogen deprivation. The high incidence of corpus luteum formation may be caused by the liberation of luteinizing hormone acting upon the pre-existing mature follicles.

Leiby [1933] found thyroid hypertrophy in spayed female rats which were injected with 100 r.u. of oestriol daily for a week. Deprivation of ovarian hormone in the rat may cause atrophic changes in the thyroid which can be restored to normal by physiological doses of oestrogen [Anderson, 1934]. In the rabbit, the injection of oestrogen produced large thyroid glands which were hyperaemic and contained abundant colloid [Karp & Rostkiewicz, 1934]. Recently, Emge & Laqueur [1941] accidentally observed that large doses of oestrone induced hyperplasia in the rat thyroid. Withdrawal of ovarian hormones by castration produced morphological evidence of decreased thyroid activity. These facts support our contention that oestrogen may stimulate the thyroid to a hyperfunctional state which suppresses the follicle-stimulating activity of the pituitary on the one hand and stimulates the luteinizing activity on the other. Our hypothesis may be illustrated by the following diagrams.



The production of both follicle-stimulating and luteinizing hormones is under the regulation of the thyroid but in reverse directions: the thyroid stimulates the secretion of luteinizing hormone and inhibits formation of follicle-stimulating hormone. The activity of the thyroid itself may be stimulated by the gonadal hormones—probably by the liberation of thyrotrophic hormone from the pituitary.

#### SUMMARY

1. Daily injection of 1 mg. of stilboestrol or oestrone into normal intact rabbits caused complete inhibition of ovarian activity for a period of 30 days; the same treatment was found ineffective in thyroidectomized animals even with a longer injection period.

2. The feeding of small doses of desiccated thyroid to thyroidectomized rabbits prevented the hypertrophy of ovarian follicles, while larger doses had an inhibitory action on the ovary.

3. The results of simultaneous administration of oestrogen and desiccated thyroid to thyroidectomized rabbits were the same as those obtained by feeding desiccated thyroid alone.

4. Small doses of oestrogen did not effectively suppress the ovarian activity in intact normal rabbits; the addition of small amounts of desiccated thyroid reinforced the inhibitory action of oestrogen.

5. Feeding small doses of desiccated thyroid to normal intact rabbits had no effect on the ovary; large doses caused incomplete inhibition of the ovarian activity.

6. The pituitary gland of the thyroid-fed normal and thyroidectomized rabbits contained a high concentration of ovulating hormone and that of oestrogen-treated animals contained very little.

7. It is concluded that the follicle-stimulating and luteinizing hormones of the pituitary are under the direct regulation of the thyroid gland.

We are greatly indebted to Dr A. S. Parkes of London who kindly supplied the ox pituitary extract and to the Organon Laboratories Ltd. for supplies of oestrone.

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# OVARIAN STIMULATION BY OESTROGENS

## 2. STIMULATION IN THE ABSENCE OF HYPOPHYSIS, UTERUS, AND ADRENAL GLANDS

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(Received 22 March 1944)

It has been suggested in discussion that the ovarian stimulation produced by oestrogen in the hypophysectomized immature rat [Williams, 1940, 1944; Pencharz, 1940] may be an indirect effect mediated through the adrenals or uterus. Bourne & Zuckerman [1942 *a, b*] have demonstrated that the adrenals play an important part in the regulation of the oestrous cycle in the rat, while the observation of Palmer & Fulton [1941] that the response to chorionic gonadotrophin is diminished in the absence of the uterus indicates that this organ may also be concerned in ovarian stimulation. The following experiments show that the adrenals and the uterus are not involved in the stimulation of the ovary by oestrogens.

### STIMULATION IN THE ABSENCE OF HYPOPHYSIS AND UTERUS

#### *Method*

A group of immature female rats was hysterectomized. A week later some were killed and the remainder hypophysectomized. Of the hypophysectomized rats, some were injected daily with 100  $\mu$ g. of stilboestrol dissolved in 0.2 ml. of sesame oil. All the rats were killed 5 days after hypophysectomy. The ovaries were dissected and weighed after fixation in Bouin's fluid and transference to 70 % alcohol and subsequently cut in serial section and stained with haematoxylin and eosin.

Five days after hypophysectomy the ovaries are significantly lighter than normal, but their normal weight is maintained or increased by a daily injection of 100  $\mu$ g. of stilboestrol [Williams, 1944].

Table 1

	Organs removed	Daily stilboestrol injection $\mu$ g.	No. of rats	Wt. of organs mg. $\pm m$	Significance of difference from control <i>P</i>
Controls	Intact	0	5	8.3 $\pm$ 0.9	—
	Uterus	0	5	10.0 $\pm$ 1.3	—
		Mean	10	9.2 $\pm$ 0.8	
Exp. 1	{ Hypophysis	0	5	5.7 $\pm$ 0.6	< 0.02
	{ Uterus	100	9	13.3 $\pm$ 1.1	< 0.01
Exp. 2	{ Adrenals	0	6	5.6 $\pm$ 0.6	< 0.01
	{ Hypophysis	100	7	9.7 $\pm$ 0.7	ca. 0.7
	{ Uterus	ca. 1000†	5	10.4 $\pm$ 1.0	ca. 0.4

† Dosage explained in text.

#### *Results*

The results in different groups are recorded in Table 1 and show that hysterectomy alone affects neither the weight of the ovaries nor the fall that normally occurs after

\* Beit Memorial Research Fellow.

hypophysectomy, and that the stilboestrol-injected rats have ovaries significantly heavier than have normal rats.

Histologically the ovaries of the stilboestrol-injected rats were indistinguishable from those of hypophysectomized rats with intact uteri given the same treatment. The number of follicles more than  $200\mu$  in diameter was obviously increased, and the majority of these follicles had no antra.

#### STIMULATION IN THE ABSENCE OF THE HYPOPHYSIS, UTERUS, AND ADRENALS

##### *Method*

A group of immature female rats was hypophysectomized and adrenalectomized 5 days after their uteri had been removed. Their drinking water was replaced by physiological saline. Half of them were injected subcutaneously with  $100\mu\text{g.}$  of stilboestrol in 0.2 ml. of sesame oil on the day of hypophysectomy and on the 4 succeeding days. All the rats were killed 5 days after hypophysectomy and their ovaries treated as in the previous experiment.

##### *Results*

In error, an overstrong solution of stilboestrol was prepared and the rats in one group in the Table were injected with  $1000\mu\text{g.}$  daily in two cases, and for the first of the five injections in the case of the other three rats. The rats in the other group were given the proper dose of  $100\mu\text{g.}$  daily.

The results give no indication that adrenalectomy had interfered with the ovarian stimulation. When the ovarian sections were examined microscopically it was found that several ovaries in both the control and injected groups were completely atrophic, all the ovarian tissue being replaced by connective tissue stroma. This is attributed to interference with the blood supply during hysterectomy and would account for the failure of the stilboestrol injections to *increase* the normal ovarian weight as they did in the previous experiment. The other ovaries, however, showed a normal atrophy in the control group and stimulation in the injected groups exactly as if the uterus and adrenals had been present.

##### SUMMARY

The ovarian stimulation produced by stilboestrol injections in immature hypophysectomized rats is unaffected by removal of the uterus, or of the uterus and adrenal glands.

There is no evidence that this effect of oestrogens is an indirect one.

I am very grateful to Prof. E. C. Dodds, F.R.S., for his interest and encouragement; to Mr I. A. Hepple for preparing the histological sections; and to the Council of the Middlesex Hospital Medical School for providing me with laboratory facilities.

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# STUDIES OF THE BIOLOGICAL ACTION OF SERUM GONADOTROPHIN

## 1. DECLINE IN OVARIAN RESPONSE AFTER HYPOPHYSECTOMY

By P. C. WILLIAMS,\* *From the Courtauld Institute of  
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(Received 22 January 1944)

Interpretation of the ovarian response to the gonadotrophin from pregnant mares' serum (serum gonadotrophin) in the intact immature rat is complicated by the secretion of endogenous gonadotrophin from the rat's own pituitary. The difficulty can be overcome by using hypophysectomized rats, but this procedure has its own disadvantages. The injection should not be given until sufficient time has elapsed after the operation to allow any circulating gonadotrophin to be destroyed. For this reason authors who normally use hypophysectomized rats give their initial gonadotrophin injection 5–12 days after hypophysectomy. At this time, however, the ovaries are atrophic and there is evidence that they are then relatively unresponsive to gonadotrophic stimulation [Williams, 1940].

I am not aware of any published experiments to determine the actual time after hypophysectomy at which a basal state of ovarian responsiveness is attained. This has been the main object of the experiment reported here.

### METHOD

Immature female rats weighing 40–60 g. were each injected with a single dose of serum gonadotrophin and killed 5 days later. Their ovaries and uteri were dissected and weighed after fixation in Bouin's fluid and transference to 70 % alcohol. The ovaries were sectioned and stained with haematoxylin and eosin for histological examination. In some cases the Fallopian tubes were also cut in serial section so that the incidence of ovulation and number of ova could be determined.

The serum gonadotrophin used was a sample of pregnant mares' serum which had been dried by the method of Greaves & Adair [1938]. The dried serum was in 15 ml. ampoules one of which was reconstituted for each series of injections by the addition of distilled water.

1 ml. of the reconstituted serum (PMS A2) was injected into each rat—a dose equivalent to 70 i.u.

Different groups of rats were injected at intervals from 4 days before hypophysectomy to 14 days afterwards.

Hypophysectomy was performed from the retropharyngeal approach under ether anaesthesia, body weight records and examination of the sella turcica at death serving as checks of the completeness of the operation.

\* Beit Memorial Research Fellow.



## RESULTS

*Ovarian weight*

The average weights of the ovaries in the groups injected at the various times before and after hypophysectomy are recorded graphically in Fig. 1 together with the

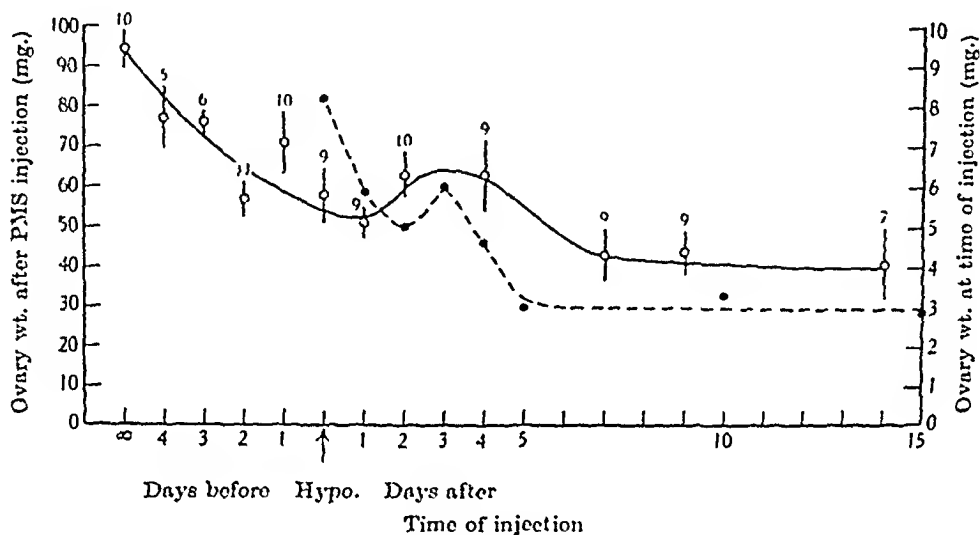


Fig. 1. O—O The ovarian weight response to a single injection of 70 i.u. of serum gonadotrophin into intact immature rats and into immature rats at intervals from 4 days before, to 14 days after, hypophysectomy. The vertical lines indicate the extent of the standard errors of the means and the figures above the number of rats in each group. ●- - -● The weight of the ovaries in intact rats and in rats at 1-14 days after hypophysectomy. There were five rats per group. The figures are taken from Williams [1944].

standard errors of the means. There was, as there usually is, considerable individual variation in the weights recorded.

*Uterine weight*

The average weights of the uteri in the different groups did not differ significantly. They ranged from 112 to 148 mg. with no trend related to the time of the injection. It is evident that 70 i.u. of serum gonadotrophin stimulates even the atrophic ovary of the hypophysectomized rat to produce sufficient oestrogen for a maximal uterine weight response.

*Ovulation and ovarian histology*

The incidence of ovulation and the average number of ova shed in those rats which ovulated are given in Table 1.

The histological changes produced in the ovaries of hypophysectomized rats by different doses of serum gonadotrophin have been described by Rowlands & myself [1941] and others, and little comment will be added here. In the intact rats the follicular apparatus was stimulated and corpora lutea were formed; luteinization of the membrana and theca was common. In the groups injected at intervals before hypophysectomy there was a gradual decline in the degree of luteinization despite wide individual variations. In the groups injected after hypophysectomy this last process

was continued; there was also a progressive decline in the number of follicles affected by the injection and a general luteinization of the interstitial tissue was noticeable.

Table 1. *Incidence of ovulation*

Interval between injection and operation	No. of rats examined	Incidence of ovulation %	Total no. of ova	Mean no. of ova per ovulation
(a) Intact controls				
—	7	71	49	10
(b) Injection before operation				
4 days	6	17	10	10
3 days	7	29	7	4
2 days	9	0	0	—
1 day				
(c) Injection at, or after, operation				
0 days	9	33	14	5
1 day	9	22	2	1
2 days	9	0	0	—
4 days	6	17	1	1
7 days	7	0	0	—
9 days	7	0	0	—
14 days	4	0	0	—

## DISCUSSION

*Ovarian weight*

Owing to individual variations in response the difference between any two adjacent points on the graph is not statistically significant, but the trend of the curve is clear and some conclusions may be drawn.

First, the fall in the first part of the curve is an index of the part played by endogenous gonadotrophin secretion in the ovarian response, the fall between two adjacent points being a measure of the effect of the gonadotrophin secreted in one day. The fall is steady within the limits of the individual variation.

Secondly, the fall in response in the period after hypophysectomy is slightly more complex. During this time two related processes are at work: a decrease in the amount of circulating endogenous gonadotrophin and the atrophy of the ovary. The atrophy of the ovary certainly plays a part in the decrease in response, since if it is prevented by stilboestrol implantation the response to serum gonadotrophin is greatly increased [Williams, 1940]. It is quite possible that the decrease in endogenous gonadotrophin, by diminishing a synergistic effect, may also lead to a decrease in response. This, however, has not been proved. An interesting factor of the curve is the hump occurring on the 2nd-4th days after hypophysectomy. I believe this has a true basis, since it coincides with a slight recovery in ovary weight which occurs in my rats on the third day after hypophysectomy. A similar recovery (on the 2nd day) has been reported by Lane & Greep [1935]. They attribute it to a release of gonadotrophin from the pituitary during hypophysectomy. The normal fall in ovary weight following hypophysectomy is shown by the dotted curve on the graph, which is constructed from figures published elsewhere [Williams, 1944]. In summary the results fully accord with the view that the initial weight and condition of the ovary play an important part in the gonadotrophic response, but the role of any endogenous gonadotrophin already in the

circulation remains uncertain. The fall in ovarian weight in the first day after hypophysectomy is not, however, reflected in any great fall in ovarian responsiveness and this may be an indication of the effect of such endogenous gonadotrophin.

Finally, a basal condition of ovarian responsiveness is attained 7 days after hypophysectomy and probably not earlier. It is unfortunate that the amount of serum available did not allow any tests to be done between the 4th and 7th days, but it seems certain that to allow 5 days to elapse between operation and injection is not sufficient. This point is of practical importance since the sooner the injection is given, the smaller will be the number of rats dying during the test.

### Ovulation

The incidence of ovulation and the number of ova shed in these intact rats are greater than in the Hampstead stock used by Rowlands & me [1941] and by Rowlands [1944]. The absence of ovulation in the groups injected 7 days or later after operation confirms our previous results.

It is interesting that ovulation could be obtained by injections up to 4 days after hypophysectomy. This may be due to circulating endogenous gonadotrophin. Rowlands & I [1943] have, however, shown that if the ovarian atrophy that follows removal of the pituitary is overcome by an injection of 40 i.u. of serum gonadotrophin given 10 days after the operation, a second injection of 40 i.u. of serum gonadotrophin given 4 days later causes ovulation. Therefore serum gonadotrophin can cause ovulation in the absence of endogenous gonadotrophin and the absence of ovulation in the groups injected later than 4 days after operation is probably due to ovarian atrophy.

### SUMMARY

A single injection of 70 i.u. of serum gonadotrophin (in the form of reconstituted dried pregnant mares' serum) was given to a group of intact immature rats and to groups of immature rats hypophysectomized at intervals from 14 days before the injection to 4 days afterwards. The rats were killed 5 days after the injection and the ovaries and uteri examined.

A basal condition of ovarian responsiveness was attained 7 days after hypophysectomy. Ovulation occurred in rats injected up to 4 days after operation but not in the rats injected later.

The roles of endogenous gonadotrophin and of the initial condition of the ovaries in the ovarian response are briefly discussed.

I am very grateful to Prof. E. C. Dodds, F.R.S., for his interest and encouragement, to Dr I. W. Rowlands for supplying the serum gonadotrophin and particulars of its assayed potency, to Mr I. A. Hepple for preparing the histological sections, and to the Council of the Middlesex Hospital Medical School for providing me with laboratory facilities.

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# STUDIES OF THE BIOLOGICAL ACTION OF SERUM GONADOTROPHIN

## 2. OVARIAN RESPONSE AFTER HYPOPHYSECTOMY AND OESTROGEN TREATMENT

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*(Received 23 May 1944)*

The ovarian response to an injection of serum gonadotrophin (pregnant mares' serum) given 10 days after hypophysectomy in the immature rat is greatly increased if the ovarian atrophy has been prevented by implanting a tablet of stilboestrol at the time of the operation [Williams, 1940]. The experiments that are described below confirm and amplify this observation.

### METHODS

#### *Animals*

Immature (40–60 g.) rats of the London Wistar strain bred in this Institute were used for all experiments.

#### *Serum gonadotrophin*

Three samples of gonadotrophin were used: PMS/A 4 was a sample of dried serum which was reconstituted by the addition of water and had a potency of 112 i.u. per ml.; PMS/IV was an extract prepared by the method of Rimington & Rowlands [1941] to the 'initial-powder' stage and contained 13 i.u. per mg.; PMS 10 was an extract whose exact history has been lost—it was only used for one minor experiment and contained about 10 i.u. per mg.

#### *Injections*

The doses were given as single subcutaneous injections except where large volumes of PMS/A 4 had to be given when five daily injections were made. In the hypophysectomized rats the injection of PMS/A 4 was given on the 10th–12th post-operative day but on the 7th day in the case of PMS/IV.

#### *Operative procedures*

Hypophysectomy was performed from the retropharyngeal approach under ether anaesthesia. Completeness of pituitary removal was confirmed by body weighings and examination of the sella turcica at autopsy.

Stilboestrol tablets made by hand-press and weighing 10–15 mg. were implanted subcutaneously in the flank immediately after the pituitary had been removed. The absorption from such tablets is about 100–150  $\mu$ g. per day [Williams, 1944a].

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*Autopsy*

The rats were killed 5 days after the first, or only, injection. Their ovaries and uteri were removed, fixed in Bouin's fluid and weighed after transference to 70% alcohol. In most cases the ovaries and Fallopian tubes were subsequently serially sectioned and stained with haemotoxylin and eosin.

Table 1. *Ovarian weights resulting from the injection of serum gonadotrophin in different types of immature rats*

Serum gonadotrophin			Weight of ovaries with no. of animals in brackets		
Sample	Dose i.u.	No. of injections	Hypo. mg. $\pm$ s.e.	Hypo. + stilboestrol mg. $\pm$ s.e.	Intact mg. $\pm$ s.e.
PMS/A 4	5.6	1	$4 \pm 1$ (6)	$12 \pm 3$ (8)	$14 \pm 2$ (5)
PMS/IV	6.5	1	—	—	$20 \pm 2$ (10)
PMS/IV	13	1	—	$81 \pm 17$ (8)	$36 \pm 4$ (10)
PMS/A 4	14	1	$6 \pm 1$ (7)	$63 \pm 6$ (6)	$30 \pm 4$ (10)
PMS/IV	26	1	—	—	$75 \pm 9$ (10)
PMS/A 4	28	1	$17 \pm 4$ (5)	$67 \pm 8$ (8)	$74 \pm 9$ (5)
PMS/A 4	56	5	$40 \pm 4$ (11)	$80 \pm 9$ (8)	$124 \pm 14$ (5)
PMS/IV	65	1	—	—	$120 \pm 8$ (10)
PMS/A 4	112	5	—	$98 \pm 23$ (6)	—
PMS/IV	130	1	—	$126 \pm 17$ (9)	$155 \pm 1$ (10)
PMS/IV	260	1	—	$104 \pm 19$ (4)	$188 \pm 12$ (9)

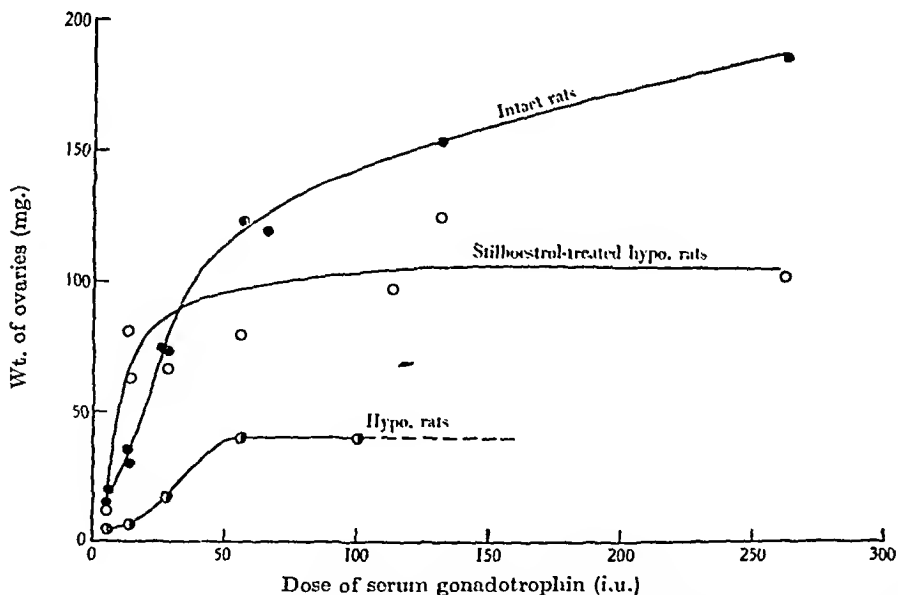


FIG. 1. Ovarian-weight response to injections of serum gonadotrophin in immature rats. ●—● Hypophysectomized rats (100 i.u. response taken from Rowlands & Williams [1941]). ○—○ Hypophysectomized rats implanted with stilboestrol. ●—● Intact rats.

## RESULTS

*Ovarian weight*

These results are collected in Table 1 and shown graphically in Fig. 1. In the hypophysectomized rats no dose higher than 56 i.u. was given; this dose produced ovaries

weighing 40 mg. which Rowlands & I [1941] have found to be the maximum weight attainable in these rats. The curve relating dose to ovarian weight in the intact rats reaches a plateau at about 190 mg. and is generally very similar to our 1941 curve. The responses in the hypophysectomized rats implanted with stilboestrol are greater than those in the ordinary hypophysectomized rats at all doses tested. With doses of 13–14 i.u. of serum gonadotrophin the response in the oestrogenized, hypophysectomized rats was even greater than that in the intact rats but with higher doses fell somewhat below it.

#### *Ovarian histology*

The findings in the intact and hypophysectomized rats duplicate those already described [Noble, Rowlands, Warwick & Williams, 1939; Rowlands & Williams, 1941; and others] but will be mentioned briefly where comparison is necessary.

In the hypophysectomized rats the lowest dose (5.6 i.u.) had no appreciable effect at all; with 14 i.u. some follicles were stimulated and there was a general luteinization of the interstitial tissue. With 28 and 56 i.u. the general appearance of the ovary was much the same as with 14 i.u.—the number of stimulated follicles was increased and some thecal luteinization had occurred but luteinization of the membrana granulosa was rare. No corpora lutea were produced.

In the intact rats even the dose of 5.6 i.u. produced apparently normal corpora lutea in some rats and the number of follicles which were stimulated and the number of corpora lutea produced were increased with the higher doses. Luteinization of the membrana granulosa was common and many of the corpora lutea were cystic with the ovum still present.

The lowest dose of serum gonadotrophin had very little effect on the stilboestrol-implanted, hypophysectomized rats although one corpus luteum was present in one of the ovaries. With higher doses the general effects were very like those produced in the intact rats whose ovaries were of the same weight: a larger proportion of the stimulated follicles did, however, appear to be luteinized and the number of follicles appeared to be larger, although their average size was smaller, than in the intact rats with ovaries of the same weight.

#### *Ovulation*

The figures in Table 2 are not intended to give an accurate quantitative picture of the incidence of ovulation in the different groups. Degenerative fragmentation of the ova makes accurate counting difficult so that the given number of ova shed may be somewhat exaggerated. Other causes of inaccuracy will be discussed later. For the present it is sufficient that the figures indicate that ovulation does occur in the oestrogenized, hypophysectomized rats. Examination of the ova suggested that those present in the tubes of the intact rats had been shed in most cases later than those in the tubes of the stilboestrol-implanted, hypophysectomized rats. The absence of ovulation in the unimplanted, hypophysectomized rats confirms previous results [Rowlands & Williams, 1941].

#### *Delayed implantation of stilboestrol*

Two experiments were carried out in which the stilboestrol tablets were implanted only when the injection of serum gonadotrophin was given. When the injection and implantation were made 10 days after hypophysectomy the oestrogen treatment had

Table 2. *Ovulation produced by the injection of serum gonadotrophin in different types of immature rats*

Serum gonadotrophin		No. of injections	No. of rats	Mean wt. of ovaries	% ovulation	Ova per ovulation
Sample	Dose i.u.					
(a) Hypophysectomized rats						
PMS/A 4	56	1	10	40	0	0
(b) Hypophysectomized rats implanted with stilboestrol						
PMS/A 4	5.6	1	6	12	0	0
PMS/IV	13	1	8	81	50	13
PMS/A 4	14	1	5	63	40	2
PMS/A 4	28	1	8	67	12	3
PMS/A 4	56	5	8	80	36	10
PMS/A 4	112	5	6	98	67	15
PMS/IV	260	1	4	104	0	0
(c) Intact rats						
PMS/A 4	14	1	6	30	83	28
PMS/A 4	28	1	5	74	60	32
PMS/A 4	56	5	5	124	80	19

no effect on the response; when they were made 7 days after hypophysectomy the response was intermediate between that of intact and hypophysectomized rats. These results are given in Table 3. Evidently the ovary is still sufficiently responsive to oestrogen stimulation 7 days after hypophysectomy for this to affect the response to gonadotrophin; after 10 days the response is too small to affect the result. This decline in ovarian responsiveness to oestrogen has been described before [Williams, 1944a].

Table 3. *Effect of delayed implantation of stilboestrol on ovarian response to serum gonadotrophin in hypophysectomized immature rats*

Animals used	Days between operation and		Mean wt. of ovaries mg. $\pm$ s.o.	No. of rats
	Inj.	Implant.		
(a) Response to 10 mg. of PMS/10				
Hypophysectomized rats	10	—	42 $\pm$ 7	4
Hypophysectomized rats implanted with stilboestrol	10	3	112 $\pm$ 28	3
	10	10	48 $\pm$ 4	5
(b) Response to 130 i.u. of PMS/IV				
Hypophysectomized rats implanted with stilboestrol	7	0	126 $\pm$ 17	9
	7	7	93 $\pm$ 10	6

## DISCUSSION

The main object of these experiments has been a qualitative one. The daily absorption from implanted tablets, particularly when they are not identical in size or weight, is variable. This factor probably accounts for the variations from the dose: response curve given in Fig. 1 for the stilboestrol-implanted rats. Then there is no experimental basis for assuming that 5 days after a single injection of serum gonadotrophin is the best time for examination of tubes to determine the incidence of ovulation or the number of ova that have been shed. Ova shed during normal ovulation [Blandau, 1943] or shed following gonadotrophin injections in hypophysectomized rats [Rowlands & Williams, 1943] pass through the tubes in 96 hr. or less and this process may

be hastened by stilboestrol implantation. Certainly any ova shed during the 24 hr. after the injection of serum gonadotrophin will have passed out of the tubes by the 120th hour.

Bearing these facts in mind the following deductions may be made. It is known that the implantation of a 10–15 mg. tablet of stilboestrol into a hypophysectomized rat at operation causes a stimulation of the ovary without significantly raising the ovarian weight. The main effect is to increase the number of medium-sized follicles, apparently by a stimulation of the membrana granulosa [Williams, 1944a]. This abnormally high number of medium-sized follicles no doubt accounts for the higher response to 13–14 i.u. of serum gonadotrophin in these rats than in normal intact rats. With higher doses the secretion of endogenous gonadotrophin in the intact rats is obviously sufficient to outweigh this abnormal responsiveness.

The incidence of ovulation in the oestrogenized, hypophysectomized rats requires further investigation, but it is clear from the results already obtained that endogenous gonadotrophin is not an essential factor in the process. I suggest that ovulation in the intact immature rats injected with serum gonadotrophin occurs both as a direct effect on the ovary and also indirectly through the presence of endogenous gonadotrophin. The direct effect probably precedes the indirect one. My rats apparently ovulate more readily than Rowlands' [1944] rats and resemble the rats used by Cartland & Nelson [1938] in this respect.

That the effects described are due to the condition of the ovaries produced by stilboestrol treatment and not to any interaction between the oestrogen and the gonadotrophin is shown by the experiments in which stilboestrol was implanted at the same time as the gonadotrophin was injected.

#### *Role of ovarian atrophy in ovarian response to serum gonadotrophin*

In intact immature rats an injection of serum gonadotrophin will lead to an increase in ovarian weight (maximally about 200 mg.), follicle stimulation, ovulation, and the formation of corpora lutea; in hypophysectomized immature rats the maximum ovarian weight attained is 40 mg. and although follicle stimulation is produced there is no ovulation or true corpus-luteum formation [see Rowlands & Williams, 1941].

These qualitative and quantitative differences have been attributed to the absence of endogenous gonadotrophin secretion in the hypophysectomized rats. (It should be noted that the fact that 40 mg. ovaries are the largest produced in the hypophysectomized rats does not explain the absence of luteinization, since ovulation and corpus-luteum formation may occur in intact rats whose ovaries weigh as little as 20 mg.) It is probable that this interpretation is true but it is certainly not the complete explanation. It is now obvious that the qualitative response of an atrophic ovary to serum gonadotrophin is quite different from that of a normal ovary. Evidence for this statement is provided by the facts that when the ovarian atrophy is overcome by an injection of 40 i.u. of serum gonadotrophin another injection of serum gonadotrophin given 4 days later will cause ovulation [Rowlands & Williams, 1943] and that prevention of the ovarian atrophy by stilboestrol implantation also permits ovulation and luteinization to occur as I have shown here. I also think that a large part of the quantitative difference is due to the ovarian atrophy—certainly the fall in ovarian responsiveness after hypophysectomy is very similar to the fall in ovarian



weight [Williams, 1944b]. The dose:response curve in the hypophysectomized, oestrogenized rats reaches a plateau at 100–120 mg. and the difference between this and the plateau at 190 mg. reached in intact rats must be attributed to endogenous gonadotrophin. This may also account for the greater incidence of ovulation in the intact rats under the conditions of the experiment. Both the gain in ovary weight and incidence of ovulation are reduced if rats injected with 70 i.u. of serum gonadotrophin are hypophysectomized between the days of injection and killing [Williams, 1944b].

On the basis of the qualitative differences between the responses in intact and hypophysectomized rats, serum gonadotrophin has been regarded as predominantly follicle stimulating. The results reported here throw doubt on such a conclusion and also on any qualitative estimate of follicle-stimulating or luteinizing potency based on the response of the atrophic ovaries of the hypophysectomized rat.

#### SUMMARY

Single injections of 5–130 i.u. of serum gonadotrophin have been made into intact, or hypophysectomized, immature rats, or into immature, hypophysectomized rats implanted with stilboestrol.

Ovulation and luteinization were produced in the intact rats and stilboestrol-implanted, hypophysectomized rats but not in the unimplanted, hypophysectomized rats.

The ovarian weight response to 13–14 i.u. was greatest in the oestrogenized, hypophysectomized rats but with higher doses the response to the intact rats was the greatest.

#### CONCLUSIONS

All the qualitative differences between the responses of intact and hypophysectomized rats to serum gonadotrophin can be attributed to the atrophic condition of the ovaries in the latter animals. Some of the quantitative differences are probably due to the same cause.

I am very grateful to Dr I. W. Rowlands for the supply of PMS/A 4 and for details of its potency; to Dr F. X. Aylward for a supply of PMS/IV; to Mr I. A. Hepple for preparing the histological slides; and to the Council of the Middlesex Hospital Medical School for providing me with laboratory facilities.

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# STUDIES OF THE BIOLOGICAL ACTION OF SERUM GONADOTROPHIN

## 3. ROLE OF ENDOGENOUS GONADOTROPHIN

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(Received 10 August 1944)

It is generally assumed that an injection of serum gonadotrophin stimulates the secretion of gonadotrophin in the immature rat since the ovarian response in intact rats is greater than, and qualitatively different from, that in hypophysectomized rats. The demonstration that ovarian atrophy seriously interferes with the ovarian response [see Williams, 1944b] suggests that further evidence is required before the assumption can be regarded as a true one. The ovarian response to 70 i.u. of serum gonadotrophin is diminished if the pituitary is removed between the time of injection and that of killing 5 days later [Williams, 1944a]. This is strong evidence that pituitary secretions play a part in the ovarian response but the dose used was high, and it is important to decide whether doses which produce ovarian weights within the normal physiological range also involve gonadotrophin secretion. Current theories to account for sexual periodicity in the female generally postulate mutual inhibitory and stimulatory actions of ovarian and hypophyseal secretions. Though such theories are probably correct, the experimental bases on which they rest are still scanty.

### EXPERIMENTAL

*Method.* Immature (40–50 g.) rats of the London Wistar strain bred in this Institute were injected subcutaneously with single doses of serum gonadotrophin (1.3–650 i.u.) and single injections of the same doses were given to similar rats 1 hr. or less after they had been hypophysectomized. Thus the responses in the intact rats could be compared with the responses in hypophysectomized rats whose ovaries were not atrophic. Five days after the injection had been given the rats were killed and the uteri and ovaries were removed and weighed after fixation in Bouin's fluid and transference to 70% alcohol. Histological sections were prepared and stained with haematoxylin and eosin. Serial sections of the Fallopian tubes were prepared in some cases.

The serum gonadotrophin used (PMS/IV) was an extract prepared by the method of Rimington & Rowlands [1941] to the 'initial-powder' stage; it contains 13 i.u. per mg.

*Results.* The increases in uterine and ovarian weights are recorded in Table 1. The figures show that the response in the intact rats was in all cases greater than in the hypophysectomized rats, as was the maximum ovarian weight attainable.

The intact rats given doses (1.3–6.5 i.u.) producing mean ovary weights of 14–20 mg. had several corpora lutea in each ovary with few or no stimulated, but unluteinized,

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Table 1. *Responses in intact and hypophysectomized rats*

Dose i.u.	Uterine wt. (mg. $\pm$ s.e.)		Ovarian wt. (mg. $\pm$ s.e.)	
	Intact	Hypo.	Intact	Hypo.
0.0	40 $\pm$ 3	20 $\pm$ 2	12 $\pm$ 1	6 $\pm$ 1
1.3	62 $\pm$ 5	18 $\pm$ 2	14 $\pm$ 1	5 $\pm$ 1
2.6	66 $\pm$ 4	19 $\pm$ 1	14 $\pm$ 1	6 $\pm$ 1
6.5	83 $\pm$ 4	37 $\pm$ 8	20 $\pm$ 2	7 $\pm$ 1
13.0	97 $\pm$ 9	76 $\pm$ 5	31 $\pm$ 3	13 $\pm$ 1
26.0	112 $\pm$ 3	141 $\pm$ 14	75 $\pm$ 9	55 $\pm$ 6
65.0	147 $\pm$ 5	117 $\pm$ 5	120 $\pm$ 8	89 $\pm$ 11
130.0	155 $\pm$ 9	133 $\pm$ 4	155 $\pm$ 11	94 $\pm$ 3
260.0	144 $\pm$ 5	152 $\pm$ 7	188 $\pm$ 12	90 $\pm$ 7
650.0*	164 $\pm$ 20	156 $\pm$ 5	176 $\pm$ 22	124 $\pm$ 9

\* 5 rats per group with this dose; otherwise 10 intact rats and 7-11 hypophysectomized rats per group.

follicles. Those given 13 i.u. had a mean ovary weight of 30 mg. and few or no corpora lutea but many stimulated ovarian follicles with no luteinization; higher doses produced follicle-stimulation both with and without luteinization. Cartland & Nelson [1938] also demonstrated a purely follicle-stimulating effect with a dose intermediate in size between doses producing corpora lutea. The hypophysectomized rats differed in that stimulated ovaries weighing less than 25 mg. never contained any corpora lutea but the heavier ovaries were similar to those of the same weight in the intact rats.

Table 2. *Incidence of ovulation*

Dose i.u.	No. of rats	Mean wt. of ovaries mg.	% ovulation	No. of ova	
				Total	Per ovulation
(a) Intact rats					
1.3	10	14	30	7	2.3
2.6	8	14	38	11	3.7
6.5	10	20	80	28	3.5
13.0	10	31	10	5	5.0
26.0	10	75	60	106	17.7
65.0	10	120	30	15	5.0
(b) Hypophysectomized rats					
26.0	9	55	22	18	9.0
65.0	11	89	64	72	10.3

The Fallopian tubes of the intact rats injected with 1.3-65 i.u. and of the hypophysectomized rats injected with 26 or 65 i.u. were serially sectioned and examined for ova. The effects of doses higher than 65 i.u. have little physiological relevance while histological examination of the ovaries was sufficient to show that ovulation had not occurred in the hypophysectomized rats injected with 1.3-13 i.u. The results given in Table 2 indicate that ovulation does occur under certain conditions in both types of rat and confirms the difference noted by histological examination of the ovaries; for ovulation does not occur in ovaries weighing less than 25 mg. in the hypophysectomized rats but does in the intact rats. The results cannot be regarded as accurate indications of the total ovulation produced in the different groups for reasons pointed out in the preceding paper [Williams, 1944*b*], but do confirm the fact already suspected [Williams, 1944*a*], that my rats are more sensitive to the ovulation-producing capacity of serum gonadotrophin than are those used by

Rowlands [1944]. Examination of the ova showed that those shed by the intact rats receiving 26 or 65 i.u. and by the hypophysectomized rats had been in the tubes for more than a day; they were showing signs of degeneration and looked like those seen in the tubes of a normal virgin rat in dioestrus. Those ova shed by the intact rats receiving 1.3–13 i.u. were obviously much more recent—in almost all cases granulosa cells still surrounded the ova which were like those seen in the tubes of normal rats during oestrus or met-oestrus.

#### DISCUSSION

The stimulated secretion of oestrogen, shown by the increase in uterine weight, and the structural stimulation of the ovaries are both greater in the intact rats than in the hypophysectomized ones. Therefore it is safe to assume that endogenous gonadotrophin plays a part in the reaction of the intact immature rat to all effective doses of serum gonadotrophin. It remains to decide whether this endogenous secretion is increased by the injection or whether the effect is purely due to the normal secretion which would occur during the 5 days of the test even if no injection had been made.

The figures in Table 1 show that 6.5–13 i.u. have to be injected to prevent any loss of uterine or ovarian weight in the 5 days after hypophysectomy, so we may conclude that the normal rate of gonadotrophin secretion in the immature rat is roughly equivalent to 10 i.u. of serum gonadotrophin in 5 days, bearing in mind that the equivalence is quantitative only. When the uterine weight and ovarian weight curves are drawn out against log. dose as in Fig. 1, it is seen that in the hypophysectomized rats the slope of the uterine weight curve is steeper than the curve in the intact ones and certainly not less steep for the ovarian weight response up to 90 mg. There is no quantitative evidence for an increased secretion of endogenous gonadotrophin in response to doses of serum gonadotrophin below 65 i.u. Once the dose used in the hypophysectomized rat is sufficient to prevent the atrophy consequent on the loss of endogenous secretion, then proportionate increases in dose produce similar quantitative changes in both intact and hypophysectomized rats. Evidence for an increased gonadotrophin secretion must, therefore, rest on the qualitative nature of the ovarian response or on the fact that the plateau of the ovarian weight curve is higher (200 mg.) in the intact rats than in the hypophysectomized rats (100–120 mg.).

The only qualitative difference between the responses in the two types of animal is the occurrence of ovulation and luteinization in intact rats with ovaries of low weight. There is no reason why this should not be the result of the normal gonadotrophin secretion. The purely follicle-stimulating action of 13 i.u. of serum gonadotrophin in the intact rats suggests that this normal secretion of luteinizing hormone is actually suppressed by this dose. There is already evidence from parabiosis experiments that an excess of follicle-stimulating hormone inhibits the secretion of luteinizing hormone [Witschi & Levine, 1934]. It is worth noting that the effect is produced here by the minimal dose of gonadotrophin necessary to produce maximal uterine weight and ovaries equal in weight to those of breeding females and so can be regarded as physiological.

The difference in the maximum ovarian weights obtained in the two groups of animals is exactly similar to that shown in the preceding paper between intact rats and hypophysectomized rats in which ovarian atrophy was prevented by oestrogen

treatment. It must certainly be attributed in some way to the gonadotrophin secretion in the intact animal, but it occurs among weights quite outside the physiological range and no detailed explanation will be attempted here. Perhaps it is an indication of an increased secretion of follicle-stimulating hormone produced at these dose levels.

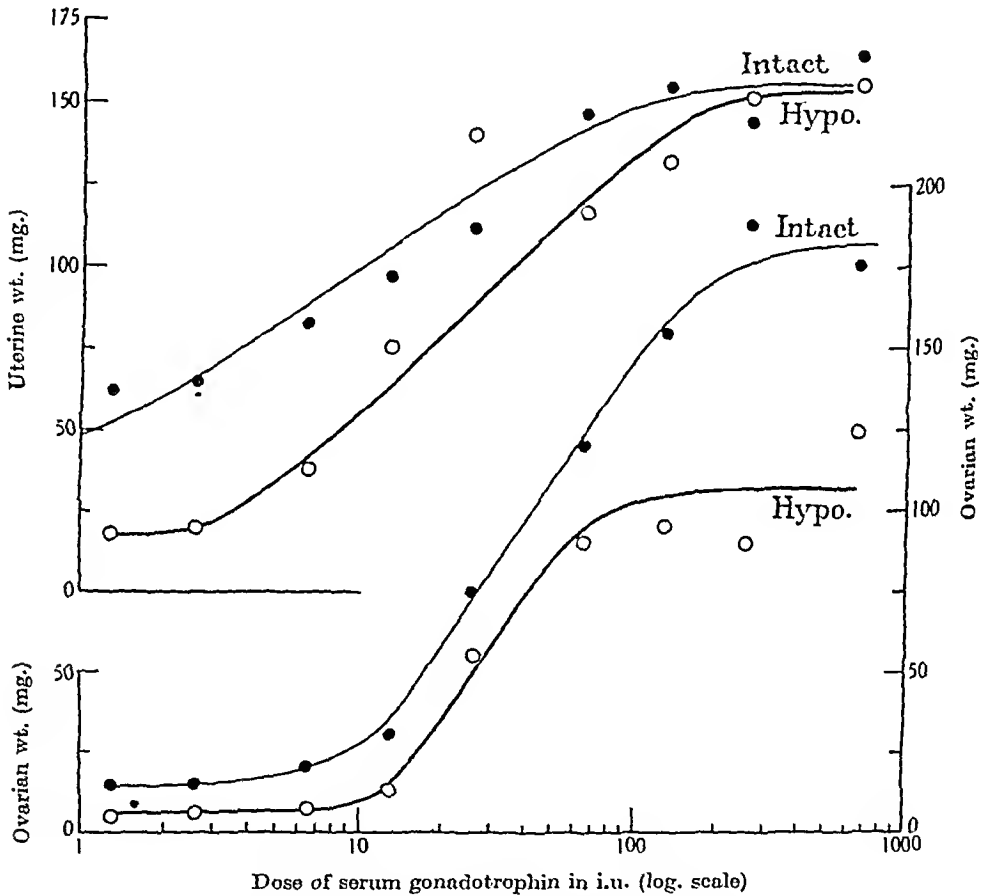


FIG. 1. Log. dose-response curve for ovarian and uterine weight responses to an injection of serum gonadotrophin in intact and hypophysectomized rats.

The response of intact immature rats to serum gonadotrophin may be interpreted in the following way. With small doses of serum gonadotrophin producing ovaries of 20–30 mg. or less, the ripening of the follicles is slow, and by the time they are ripe the concentration of injected gonadotrophin is insufficient to produce ovulation and luteinization—these effects are, however, produced by endogenous gonadotrophin. The secretion of the latter is suppressed by higher doses of serum gonadotrophin, but these doses either ripen the follicles faster or are large enough to ensure that an adequate concentration of gonadotrophin will be present when the follicles are ripe. In the present experiment the follicles were presumably ripened faster, since ovulation occurred earlier in the rats injected with the higher doses of serum gonadotrophin. In the intact rats referred to in the preceding paper, ovulation was just as recent with the higher doses as with the lower, so that no generalization can be made on this

point. It may be that there are qualitative differences between the extract PMS/IV used here and the dried serum PMS/A4 chiefly used in the other experiment, or there may have been some seasonal change in the responsiveness of the rats. The general interpretation, however, still holds. Whether this means that serum gonadotrophin is 'predominantly follicle-stimulating' becomes a matter of definition.

Lane & Greep [1935] suggested that extra gonadotrophin was released from the pituitary during removal of the gland. In the operation as I carry it out, the rupture of the dura leads to flow of cerebro-spinal fluid and blood through the burr-hole, where suction is immediately applied. It is difficult to see how any abnormal amount of gonadotrophin could be released into the circulation at this time and I have been unable to demonstrate any increase in uterine or ovarian weight in immature rats that have had their pituitaries traumatized without any removal of blood or cerebro-spinal fluid. Nor do I think that there is any serious evidence that the endogenous gonadotrophin already present in the circulation at the time of operation is sufficient to interfere with the qualitative action of injected serum gonadotrophin. Certainly a test carried out 1 hr. after hypophysectomy will give a more accurate picture of the activity of a sample than will a test made 7 days later as well as giving a steeper and more extensive dose-response curve.

#### SUMMARY

Doses of 1-650 i.u. of serum gonadotrophin were injected into intact immature female rats and into similar rats 1 hr. after hypophysectomy.

In the intact rats doses of 13 i.u. were purely follicle-stimulating: ovulation and luteinization were produced with lower or higher doses. The maximum ovarian weight attainable was 180-200 mg.

In the hypophysectomized rats the maximum ovarian weight was 100-120 mg. Histologically the ovaries were similar to those of the same weight in the intact rats except that ovulation and luteinization were not obtained with ovaries weighing less than 25 mg.

The log. dose-response curves for uterine or ovarian weight are parallel in the two groups.

There was loss of ovarian and uterine weight in the hypophysectomized rats injected with less than 10 i.u. and this dose is therefore regarded as quantitatively equivalent to the normal secretion of endogenous gonadotrophin in 5 days.

The ovulation and luteinization produced in intact rats with ovaries of less than 25 mg. must be due to endogenous gonadotrophin; that produced with higher doses in intact and hypophysectomized rats is due to the injected gonadotrophin alone.

#### CONCLUSIONS

There is no evidence for an increased secretion of endogenous gonadotrophin following injections of serum gonadotrophin giving responses within the normal physiological range. It is suggested that the response in rats immediately after hypophysectomy is a more accurate indication of the intrinsic activity of injected gonadotrophin than the responses in intact rats or in rats injected when ovarian atrophy has occurred following hypophysectomy.

I am very grateful to the Council of the Middlesex Hospital Medical School for providing me with laboratory facilities; to Dr F. X. Aylward for the PMS/IV; and to Mr I. A. Hepple for preparing the histological sections.

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# ADRENALECTOMY AND GASTRIC SECRETION

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(Received 17 August 1944)

In the course of a series of dissections, it was noted that the pH of the stomach contents of adrenalectomized rats was uniformly roughly neutral. This finding suggests that adrenalectomy involves an interference in gastric secretion. There is extant a considerable literature on the relation of the adrenal cortex and the processes of resorption, though little is known concerning the possible relation of the adrenal cortex and the processes of secretion. The present paper describes an investigation of the effect of adrenalectomy on gastric secretion.

## METHODS

Male rats weighing 110–140 g. were used. Adrenalectomy was performed under ether narcosis. The adrenalectomized animals received Rubin-Krick solution to drink instead of water, and a daily subcutaneous injection of 2 ml. of a solution of 1.4 % NaCl + 0.6 % NaHCO<sub>3</sub>. The stomach contents were examined 2–5 days after operation. Animals intended for examination received no food during the preceding night but were allowed access to Rubin-Krick solution.

An initial series of experiments was carried out according to the method of Roe & Dyer [1939]. The abdomen was opened under amytal narcosis, the duodenum being ligated near the pyloric sphincter. Doryl was administered a quarter of an hour later subcutaneously in a dose of 8  $\mu$ g. per kg. body weight. Samples of the stomach juice were removed by syringe after intervals of  $\frac{1}{2}$ , 1, 2, and 3 hr.

In a second series of experiments the duodenum was ligated under light ether narcosis, the abdominal cavity then being closed. After an hour, Doryl was administered subcutaneously in a dose of 8–10  $\mu$ g. per kg. body weight. An hour later the animals were killed. The stomach was removed, freed from blood by bathing in saline, and its contents carefully collected. Acid was determined on samples of 0.1–0.5 ml. of gastric juice by titration with *N*/20 or *N*/50 NaOH. The findings are expressed as ml. *N*/10 HCl per 100 ml. gastric juice.

pH was estimated with the aid of universal indicator paper (Merck). Rennin was determined by the method of Michaelis & Rothstein [1920], as was pepsin in the earlier experiments, though in later experiments the method of Anson & Mirsky [1932] was used.

## RESULTS

### *Disturbance of acid secretion in adrenalectomized rats*

The pH of the stomach contents ranged between 5 and 7 in adrenalectomized rats and between 1 and 2 in normal control animals (eleven experiments).

The results of experiments carried out by the method of Roe & Dyer [1939] are set forth graphically in Fig. 1.



In the second series of experiments the rats were killed and the gastric contents collected an hour after the Doryl injection so that interference from shock was minimized. The results are presented in Table 1.

In the adrenalectomized group two animals gave almost normal responses with values of 55 and 64 for free acid, 95 and 100 for total acid, and .4 and 5.5 ml. for volume of secretion. These figures are incorporated in the averages but not in the maximum and minimum figures of Table 1.

In a further experiment, the ability of gastric mucosa to excrete neutral red was examined. Two normal and two adrenalectomized rats were given 1 ml. of a 1 % solution of neutral red intravenously as directed by Morrison [1938-9] simultaneously with the injection of Doryl. After 1 hr. the stomach contents were carefully removed by means of a syringe. The gastric juices of the two normal animals were of a deep red colour, and normal in acidity and volume. The gastric juices of the two adrenalectomized rats failed to show red coloration (even when acidified with HCl), contained no free HCl, and only 18-20 of total acid, the volume of the secretion being 0.4 ml. Whereas in the two normal animals the mucosae of the entire alimentary tract and the pancreas were diffusely stained with neutral red, no definite red coloration was observed in the alimentary tract and pancreas of the adrenalectomized rats.

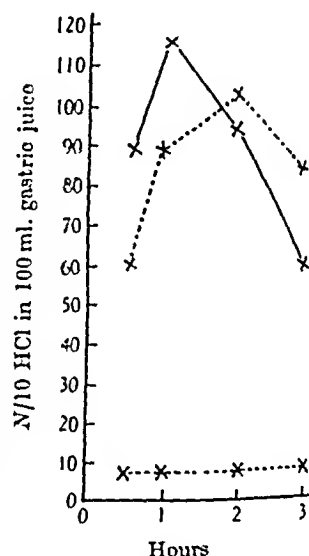


FIG. 1. Gastric secretion of acid by adrenalectomized and normal rats injected with Doryl (8  $\mu$ g. per kg.). — Normal rats. ---- Adrenalectomized rats. ..... Adrenalectomized, cortin-treated rats.

Table 1. Acid content of the gastric secretion of adrenalectomized rats injected with Doryl

Treatment	No. of animals	N/10 HCl in 100 ml. gastric juice		Volume of gastric secretion ml.	pH of gastric secretion
		Free acid	Total acid		
Adrenalectomized	26	5 (0-26)	24 (6-50)	1.1	6-8*
Controls	23	54 (20-85)	97 (60-135)	3.6	1-2†

\* In two cases pH values outside the tabulated range and rating 3 and 5 respectively were encountered.

† In two cases pH values outside the tabulated range and rating 4 and 5 respectively were encountered. Unbracketed figures in the table are averages. Bracketed figures give the maximum and minimum values found.

#### *Disturbances in secretion of gastric enzymes in adrenalectomized rats*

**Rennin.** Rennin was determined by the method of Michaelis & Rothstein [1920]. The gastric juice was diluted tenfold and roughly neutralized to litmus. Reaction mixtures contained in a total volume of 5 ml. the following amounts of gastric juice: I, 0.1 ml.; II, 0.05 ml.; III, 0.025 ml.; IV, 0.0125 ml.; V, 0.0067 ml., and milk clotting times were determined. The results are presented in Table 2.

**Pepsin.** Peptic activity was determined in a preliminary experiment by the Michaelis & Rothstein [1920] method which compares the clarification of a protein solution by different concentrations of gastric juice and by a standard pepsin solution.

Table 2. *Rennin content of the gastric juice of adrenalectomized rats injected with Doryl*

Adrenalectomized						Controls					
Clotting time in min. Dilution						Clotting time in min. Dilution					
No.	I	II	III	IV	V	No.	I	II	III	IV	V
362	2	—	—	—	—	368	2	2	2	—	—
363	30	—	—	—	—	369	1½	2	5	10	25
398	60	90	—	—	—	370	1½	2½	3	6	12
500	60	—	—	—	—	513	Imme- diately	½	2½	4½	14
502	70	135	—	—	—	516	Imme- diately	½	1	3½	6

The dilutions were made up to contain gastric juice in the following amounts: I, 0.5 ml.; II, 0.25 ml.; III, 0.125 ml.; IV, 0.067 ml.; V, 0.033 ml. The results are given in Table 3.

Table 3. *Peptic activity of the gastric juice of adrenalectomized rats after Doryl injection*

Animal	Clarification in dilution					After
	I	II	III	IV	V	
	Adrenalectomized					
429	—	—	—	—	—	12 min.
	++	+	(+)	—	—	18 hr.
440	—	—	—	—	—	30 min.
	—	—	++	(+)	—	20 hr.
445	—	—	—	—	—	15 min.
	+	(+)	—	—	—	30 min.
	Controls					
430	+	+	—	—	—	12 min.
	++	++	++	++	+	18 hr.
449	++	++	+	—	—	30 min.
	+++	+++	+++	++	+	20 hr.
360	++	+	—	—	—	15 min.
(Medullectomized)	++	++	+	+	+	30 min.

+ = clarification as by standard pepsin solution.

++ = clarification greater than that induced by standard pepsin solution.

+++ = clarification decidedly greater than that induced by the standard pepsin solution.

(+) = slight clarification effect.

Following the demonstration by means of the Michaelis-Rothstein method of a difference in peptic activity between gastric juice of adrenalectomized and normal rats respectively, measurements of greater accuracy using the method of Anson & Mirsky [1932] were carried out. The results are given in Table 4 and expressed as pepsin units (P.U.) of Anson & Mirsky.

When Doryl was not given, the gastric juice analyses then showed: in four normal animals averages of 18 of free acid, 65 of total acid, 2.6 ml. of volume of gastric juice, and 0.00234 P.U. of peptic activity; in fifteen adrenalectomized rats, averages of 8 of free acid, 34 of total acid, 0.9 ml. of volume of gastric juice and 0.00182 P.U. of peptic

Table 4. *Peptic activity of the gastric juice of adrenalectomized rats after injection of Doryl*

Adrenalectomized		Controls	
Animal	P.U.*	Animal	P.U.*
461	0.00138	424	0.00247
462	0.00219	468	0.00254
463	0.00172	425	0.00308
502	0.00107	476	0.00337
544	0.00171	513	0.00244
Average	0.00161	Average	0.00278

\* P.U. = pepsin units of Anson &amp; Mirsky.

activity. These values correspond in trend to those observed after Doryl injection, but the differences between normal and adrenalectomized animals are less pronounced.

*Stimulation of gastric secretion by insulin and histamine in adrenalectomized rats*

The results of experiments comparing the effect of histamine and insulin on gastric secretion in normal and adrenalectomized rats are assembled in Table 5.

Table 5. *Effect of insulin and histamine on gastric secretion in adrenalectomized rats*

Animal no.	Injection	Acidity in N/10 HCl in 100 ml. gastric juice		pH	Vol. of secretion ml.	Pepsin r.u. of Anson- Mirsky	Rennin
		Free acid	Total acid				
Adrenalectomized rats							
458	0.2 unit insulin	0	20	6.7	0.5	0.00122	—
460	Do.	10	40	4	0.7	—	—
518	Histamine 3 $\mu$ g.	0	18	7	0.35	0.00266	—
522	Do.	0	18	6.7	0.40	0.00216	Coagulation after 40 min. in I*
524	Do.	0	14	7-8	0.60	0.00090	—
Control rats							
431	0.2 unit insulin	56	85	1	6.0	0.00279	—
420	Do.	78	118	1	4.0	0.00254	—
437	Do.	100	125	1	3.5	0.00275	—
485	Histamine 3 $\mu$ g.	68	125	1	5.5	0.00271	Coagulation after 8 min. in I-V*
519	Do.	48	98	1	3.2	0.00185	Coagulation after 8 min. in I-V*

\* Dilutions as in Table 2.

*Differences in stomach digestion between normal and adrenalectomized rats*

In view of the differences between normal and adrenalectomized rats as to HCl and enzyme secretion, samples of food digested *in vivo* by normal and adrenalectomized rats were compared. The animals were fasted for a day, the duodenum being ligated on the following morning in the usual manner. After a rest period lasting 1-2 hr., 1.5-2 ml. of milk were administered by stomach tube. 1-1½ hr. later the rats were killed and the contents of their stomachs collected. The volume of the gastric

juice varied between 3 and 4 ml. in five adrenalectomized, and between 5 and 6 ml. in five normal animals. The average pH was 6-7 in the adrenalectomized and 1-3 in the normal animals. The stomachs of the normal rats contained only traces of undigested milk, whereas the stomachs of the adrenalectomized animals contained milk in clotted, but largely undigested lumps.

No difference in gastric motility between normal and adrenalectomized rats was detectable roentgenologically after administration of barium sulphate.\*

*Mucin content of the gastric secretion of adrenalectomized rats*

The gastric secretion of adrenalectomized rats is frequently of a translucent and mucous or threadlike consistency. In some experiments the mucin content of the gastric secretion was determined. The mucin was precipitated with 4 vol. of 96 % alcohol and the suspension allowed to settle overnight and packed by centrifugation. The precipitate was then hydrolysed for 1 hr. in 5 % HCl on a steam-bath. Mucin was determined as glucose in the neutralized hydrolysate according to the method of Somogy. The results are presented in Table 6.

Table 6. *Mucin values of gastric juice*

Animal	Treatment	Mucin as glucose mg./ml.	Animal	Treatment	Mucin as glucose mg./ml.
<b>Adrenalectomized rats</b>					
522	Histamine	0.93	485	Histamine	0.056
550	Doca + Doryl	0.869	562	Doryl	0.077
543	Doca + Doryl	0.539	559	Doryl	0.112
555	NaCl + Doryl	0.898			
558	Doryl	0.770			
<b>Adrenalectomized animals after Cortin treatment with normal HCl values:</b>					
523	Doryl	0.086			
542	Doryl	0.138			

*Influence of traumatic shock on gastric secretion*

Shock is induced with relative ease in adrenalectomized animals by narcosis and laparotomy. It seemed possible that shock might be responsible for the observed deficiency of gastric secretion in adrenalectomized rats. To test this point, normal animals were exposed to severe shock. The stomachs were ligated under amytal narcosis. Doryl was injected, and in the next hour the intestines were massaged three times for 2 min. at a time. Throughout this period the animals were maintained under narcosis. The temperatures fell to 33-34° C. An abundant transudate accumulated in the abdominal cavity. Examination of the gastric secretion yielded the figures given in Table 7.

In a second shock experiment the thighs of an animal were broken under ether narcosis shortly before the injection of Doryl. This method of shock, too, did not affect the gastric secretion. The stomach content of the animal was found to contain 50 free acid and over 100 of total acid. The findings corroborate the results of Necheles & Olson [1941], who found traumatic shock to be without influence on gastric secretion in the dog.

\* We are much obliged to Dr Shorr of the X-ray Institute of the Hadassah-Rothschild University Hospital for his help with these experiments.

Table 7. *Gastric secretion in traumatic shock*

Animal	Acidity of gastric juice in N/10 HCl on 100 ml.		Volume of secretion	Pepsin (r.u.)	Rennin coagulation time
	Free acid	Total acid			
388	70	95	1.0	—	—
394	65	102	1.0	—	After 4½ min. in I-V
393	37	65	0.25	—	—
475	78	123	1.1	0.00412	After 9 min. in I-V

*General effects of vagus stimulation in adrenalectomized rats*

The general effects of vagus stimulation following administration of increased Doryl dosages are the same in adrenalectomized and normal rats, except that no increase in gastric secretion occurs in the former. An adrenalectomized and a normal rat were given a twenty-fold dose of Doryl (250  $\mu$ g. per kg. body weight). After 2 min. both animals showed noticeable effects of vagus stimulation, such as depressed pulse, micturition, and abundant secretion from nose and eyes. Gastric secretion in the adrenalectomized animal, however, was unaffected. The gastric juice of the adrenalectomized animal after 1 hr. was about 0.7 ml. in volume, slimy in consistency, contained no free acid, and only 20 total acid. The pepsin value was 0.00071 r.u. The rennin content was slightly increased, coagulation in dilution V being observed after 20 min.

Esmodil in a dose of 800  $\mu$ g. per kg. body weight induced in adrenalectomized as well as in normal rats immediate reduction of the pulse rate to 140 per min., enhanced secretion of saliva and abundant lachrymal secretion. The lachrymal fluid of both normal and adrenalectomized rats contained porphyrin from the Harderian gland [Figge & Salomon, 1942]. The acid values of the gastric secretion were: free acid 0, total acid 25.

*Gastric secretion in medullectomized rats*

Three medullectomized rats resembled normal rats as to free acid, total acid, and enzyme content of their gastric secretion.

*Influence of increased NaCl administration or deoxycorticosterone acetate on gastric secretion in adrenalectomized rats*

Rats in this test group received 2 ml. of a solution of 1.4 % NaCl + 0.6 % NaHCO<sub>3</sub> by subcutaneous injection on the evening before examination. Four further injections were administered at intervals of 1 hr. on the following day until the rats were killed. The findings are given in Table 8.

Adrenalectomized rats received 1 mg. of deoxycorticosterone acetate when placed under fast, and a further dose of 1-4 mg. on the following day. A slight increase in the volume of the gastric secretion and a higher rennin content were observed. The treatment did not, however, improve secretion of acid and pepsin. The mucin content of the gastric juice remained high. The findings of these experiments are also given in Tables 6 and 8.

Table 8. *Gastric juice of adrenalectomized rats treated with NaCl or deoxycorticosterone acetate*

No. of expts.	Acidity of gastric juice in N/10 HCl per 100 ml.		Vol. of secretion ml.	pH	Mucin (mg. glucose per ml.)	Pepsin p.u.	Rennin
	Free acid	Total acid					
(a) NaCl treatment							
5	2	22	1.55	6-7	0.533	0.00116	No coagulation in V after 45-80 min.
(b) Deoxycorticosterone acetate treatment							
9	3	28	2.0	6	—	0.00137	Coagulation from I-V between 15-90 min.

*Influence of adrenal cortex extract (Upjohn's) on gastric secretion in adrenalectomized rats*

In the first four experiments the rats received 2 rat units at the beginning of the fast period and an additional unit on the next morning, 1 hr. before operation. In later experiments the rats received 2.5-4 rat units of adrenal cortex extract on the evening before, and 3-4 injections of 1.2-2.5 rat units at hourly intervals on the day of experiment. The results are presented in Table 9.

Table 9. *Gastric secretion in response to Doryl in adrenalectomized rats given Upjohn's adrenal cortex extract*

Animal	Cortin administration		Acidity of gastric juice in N/10 HCl per 100 ml.		Vol. of secretion ml.	pH	Pepsin p.u.	Rennin coagulation time
	No. of doses	Total dose rat units	Free acid	Total acid				
525	2	3.2	25	100	1.5	4	0.00183	In 5 min. in I-V
523	2	3.0	45	95	1.8	1	0.00251	In 13 min. in I-V
526	2	3.0	*	63	1.0	*	—	In 18 min. in I-III
527	2	5.0	0	25	0.3	6	—	—
533	5	14.0	65	110	2.6	1	0.00172	In 3 min. in I-V
535	6	8.7	*	95	2.2	*	0.00298	In 3 min. in I-V
538	5	11.2	*	52	4.5	4	0.00111	In 15 min. in I
545	5	7.5	52	88	2.4	1	0.00231	In 6 min. in I-V
542	5	7.5	30	70	2.1	1	0.00168	In 19 min. in I-V
Average			36	77	2.0	2	0.00202	

\* Because of contamination of the sample with blood or faeces, free acid and pH could not be determined.

## DISCUSSION

Our experiments show that adrenalectomized rats have a disturbed gastric secretion which conforms in type to achylia gastrica. The disturbance is not mediated by the operative shock to which adrenalectomized rats are more susceptible, since artificially induced shock fails to affect gastric secretion. Moreover, the induced achylia is not mediated by a change in vagus excitability, since Doryl or Esmodil in a suitable dosage produces symptoms of vagus stimulation but no secretion of gastric juice. Deficiency in adrenal medulla alone does not affect gastric secretion. The disturbances

in salt balance which follow adrenalectomy cannot be the cause of the observed gastric disturbance, since the latter is not affected by administration of sodium chloride in an amount sufficient to counterbalance any induced salt deficiency. Administration of deoxycorticosterone acetate and sodium chloride together was also without regular influence on the gastric secretion, though rennin secretion, surprisingly, was favourably affected by the deoxycorticosterone treatment.

It should be emphasized that disturbances in resorption due to adrenal deficiency disappear following administration of deoxycorticosterone [Stein & Wertheimer, 1941]. The disturbance in gastric secretion evinced by adrenalectomized rats, on the other hand, could only be cured in our experiments by the administration of a whole cortical extract, e.g. Upjohn's adrenal cortex extract.

Thaddea [1936] described disturbances of gastric secretion in Morbus Addison and found marked reduction or cessation of acid production in advanced stages of this condition. Administration of cortical hormone led to marked improvement in the acid secretion. It would thus appear that further investigation of gastric secretion in Morbus Addison may have both a diagnostic and therapeutic interest.

A disturbance by adrenalectomy of a secretory function other than gastric secretion has been described recently. Gaunt, Eversole & Kendall [1942] have shown that lactation is diminished or abolished by adrenalectomy and that this inhibition is also unaffected by deoxycorticosterone acetate. On the other hand, administration of whole cortical extracts or of compound E of Kendall restored normal lactation. Compound A of Kendall was almost equally effective.

It is of further interest that the secretory activity of the kidney tubules is stimulated by compound E, but is not influenced by compound A [Chambers & Cameron, 1944]. It may be tentatively suggested that secretion, whether of gastric juice, milk, or from the renal tubules, depends on energy-producing processes for whose realization cortin is essential. While the same adrenal hormone which influences carbohydrate metabolism appears also to govern different secretory functions, the latter are not affected by cortical hormones which influence electrolyte metabolism.

#### SUMMARY

1. Adrenalectomized rats in good general condition show marked diminution of gastric secretion following stimulation with Doryl. Gastric juice of such rats contains little or no free acid, only small amounts of total acid, is low in enzyme content, and rich in mucin.

2. Medullectomy alone does not depress gastric secretion.

3. Artificial shock does not disturb gastric secretion.

4. Increased intake of sodium chloride and treatment with deoxycorticosterone acetate do not restore the gastric secretory function of adrenalectomized rats.

5. Following administration of adrenal cortex extract (Upjohn) normal gastric secretion is restored.

#### CONCLUSION

Different secretory functions—gastric secretion, lactation, renal-tubular secretion—depend on the adrenal hormone which governs carbohydrate metabolism.

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# 'GROWTH' IN RELATION TO THE DIABETOGENIC AND PANCREOTROPIC ACTIONS OF ANTERIOR PITUITARY EXTRACT

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(Received 25 August 1944)

Young [1941], on the basis of his experimental work with dogs, has suggested that the pre-diabetic increase of height and weight in children and adults respectively is due to excessive function of the anterior pituitary gland compensated by increased activity of the pancreatic islets, and that failure of this balanced mechanism from inability of the islets to maintain their necessarily overactive condition ultimately results in diabetes mellitus. The purpose of the present paper is to adduce further experimental evidence in favour of such a theory.

## METHODS

*Extract.* A crude saline extract of fresh ox anterior pituitary glands was prepared after the method of Young [1938], so that 2 ml. were equivalent to 1 g. of gland. The extract was stored at a low temperature without freezing, used within 6 days of preparation, and injected by the subcutaneous route. The injections consisted either of a constant amount of 1.5 g. of gland per kg. body weight or of a quantity which was increased by 0.5 g. of gland per kg. at intervals of 5 or 6 days from an initial 1 g. of gland per kg. to a final 2.5 g. of gland per kg. body weight.

*Animals.* The animals investigated were English rabbits, eight males and seven females, and weighed between 1615 and 2211 g., averaging 1983 g. They were kept in metabolism cages and given daily 100 g. of a mixture of 40 % oats, 30 % bran and 30 % maize, 300 g. cabbage, 25 g. hay (four animals only), and water *ad lib.* The energy value of this diet was calculated by analysing its constituents as regards carbohydrate, protein and fat and applying the usual factors  $4.1 \times 9.3$ . Daily measurements included body weight, food consumption, urinary volume, and, when present, urinary sugar and ketones. The ten control rabbits used to estimate the pancreatic islet tissue were also English, seven males and three females, and weighed between 1530 and 2380 g., averaging 1947 g.

*Estimations.* Urinary sugar was estimated by Cole's method, urinary ketones by the Van Slyke-Denigès method, and the pancreatic islet tissue after the method described by Ogilvie [1937]. The alpha and beta cells of the islet tissue were differentially stained by Heidenhain's haematoxylin.

## RESULTS

### *Body weight, food value, and urinary volume*

The fifteen animals during a control period of 10 days maintained an almost constant body weight and urinary volume on a practically fixed food value. Treated with anterior pituitary extract, they either increased, remained constant or decreased in weight and were accordingly divided into groups 1, 2 and 3, details of which and of

the entire series are shown in Table 1 and Figs. 1 and 2. The increase and maintenance of weight in groups 1 and 2 took place, incidental to a loss of appetite, on much less than the control calorie requirement, while the diminution of food value in group 3 was definitely more marked than the loss of weight. The series as a whole

Table 1

	Group 1	Group 2	Group 3	Entire series
No. of animals	7	4	4	15
Duration of treatment	15 days	10 days	10 days	12 days
Average amount of A.F.G.* per animal	43.6 g.	31.5 g.	28.6 g.	36.3 g.
Body weight: Average per day	+7.3 g.	$\pm 0$ g.	-12.1 g.	+1.8 g.
Total	+5.7 %	$\pm 0$ %	-5.9 %	+1.1 %
Average caloric intake per day relative to control	65 %	65 %	39 %	58 %
Average urinary volume per day relative to control	87 %	90 %	47 %	77 %
Glycosuria: No. of animals	7	4	4	15
Duration	9 days	9 days	10 days	9 days
Maximum	9.6 g./day	16.0 g./day	2.4 g./day	9.4 g./day
Ketonuria: No. of animals	5	3	4	12
Duration	6 days	3 days	6 days	5 days
Maximum	757 mg./day	109 mg./day	340 mg./day	456 mg./day

\* Anterior pituitary gland.

maintained or even slightly increased its weight on rather more than half the necessary control food value. Each group and the entire series for the most part by temporarily excreting sugar and ketones lost some energy and must consequently have had an even more reduced caloric intake than their accredited amount. The glycosuria and body weight, it might here be noted, showed an inverse relationship in four animals. Further, as the urinary volume in each group and therefore in the series as a whole was less markedly reduced than the food value, the fall in caloric intake was in general accompanied by a relative polyuria. This phenomenon, which was probably due to the consumption of relatively more green food than bran mixture and not to the drinking of water, in turn favours the conclusion that fluid retention played no part in the actual or relative increase of weight in the various groups and entire series on a reduced food value. As seen in Fig. 2, the average weight of the series after treatment fell on a greater caloric intake than that obtaining in the injection period, while the urinary volume, probably owing to the observed drinking of water at this time, was increased to an actual polyuria.

#### *Pancreatic islet tissue*

The weight of islet tissue and the average weight and number of the islets in the fifteen injected rabbits and also in ten control animals are given in Table 2. The injected series, it is evident, had on the average more than twice as much by weight of islet tissue as the control group, and the islets of the injected animals compared with those of the control rabbits were on the average more than twice as much by weight (Fig. 3) and within normal range as regards number. The normality of the

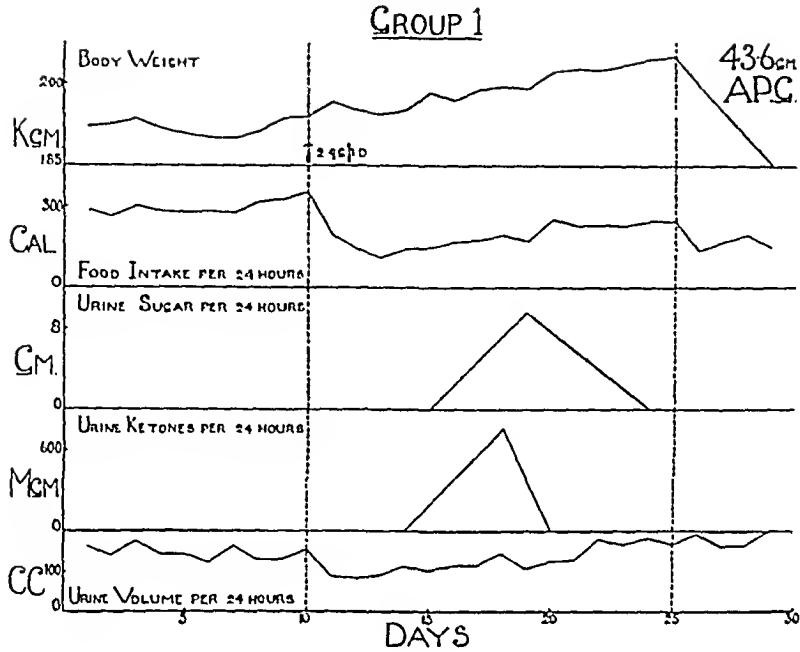


FIG. 1. Each of the seven rabbits upon the mean of which this figure is based received an average of 2.9 g. of anterior pituitary gland per day (total 43.6 g.) between the 10th and 21st day inclusive. The treatment resulted in increase of the body weight, reduction of the caloric intake, transitory glycosuria and ketonuria, and relative polyuria.

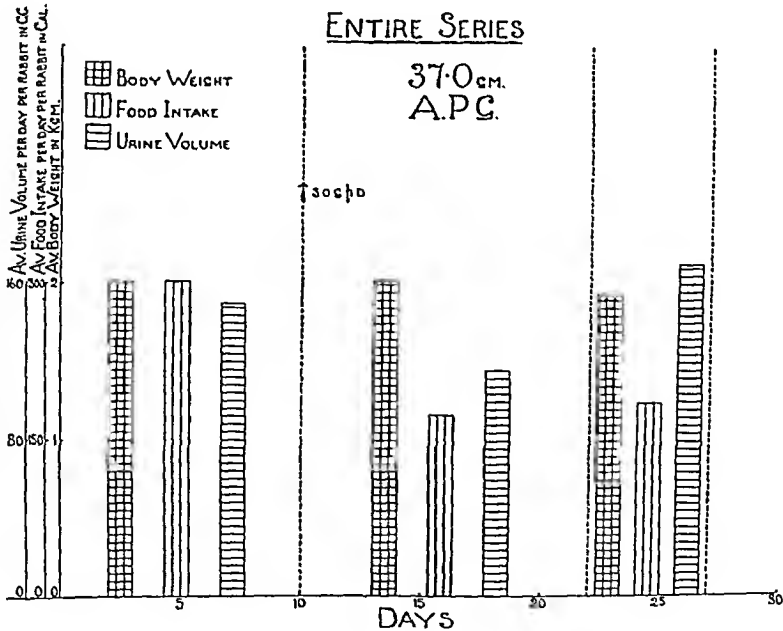


FIG. 2. Each of the fifteen rabbits upon the mean of which this figure is based received an average of 3.0 g. of anterior pituitary gland per day (total 37.0 g.) between the 10th and 21st day inclusive. The treatment resulted in maintenance or even slight increase of the body weight, reduction of caloric intake and relative polyuria.

islets in number was corroborated by the fact that the intralobular pancreatic ducts which ordinarily proliferate before giving rise to new islets were normal in number, distribution and structure. Finally, the islets of the injected animals apart from their increased size were normal architecturally and in their proportion of alpha and beta cells.

Table 2

	No. of rabbits	Wt. of islet tissue g. $\pm$ S.E.	Average wt. of islets $\mu$ g. $\pm$ S.E.	(No. of islets $\pm$ S.E.) $\times 10^{-3}$
Injected	15	0.113 $\pm$ 0.026	0.494 $\pm$ 0.053	218 $\pm$ 34
Control	10	0.053 $\pm$ 0.006	0.240 $\pm$ 0.028	238 $\pm$ 27



Fig. 3. Comparison of islet tissue in injected and control rabbits. The average weight (0.496  $\mu$ g.) of the upper group of forty-seven islets from the body of the pancreas of injected rabbit 12 approximates closely to the average weight (0.494  $\mu$ g.) of the islets of the entire injected series, while the average weight (0.235  $\mu$ g.) of the lower group of fifty-three islets from the head of the pancreas of control rabbit 4 approximates closely to the average weight (0.240  $\mu$ g.) of the islets of the entire control series. The islets of the upper group are on the average more than twice the size of those in the lower group.

## DISCUSSION

The animals in this investigation responded to treatment with crude anterior pituitary extract by increasing actually or relatively in weight on a definitely smaller caloric intake than that normally required for the maintenance of constant body weight. Such an observation is in agreement with the results of previous investigators. Thus, Lee & Schaffer [1934] and Lee [1938] found that when restricted to the same food intake normal rats treated with anterior pituitary extract gained significantly more weight than controls. The same finding was obtained in hypophysectomized rats by Marx, Simpson, Reinhardt & Evans [1941-2], who also noted that the internal organs except the thymus grew at approximately the same rate as the body as a whole. Again, Young [1941-2, 1942] has shown that on a constant daily amount of food just sufficient to maintain its body weight a normal dog or cat treated with pituitary extract increases in weight despite the occurrence of glycosuria and may show extensive deposits of fat after death. These investigations and the present one

thus justify the conclusion that anterior pituitary extracts reduce the metabolic rate, although not necessarily the basal metabolic rate, and probably also increase anabolism.

Both the reduced metabolic rate and increased anabolism are attributable to known actions of anterior pituitary extract. Thus, the oxidation of carbohydrate as emphasized by Russell [1938] is suppressed by its diabetogenic property, while an equally important effect according to Mirsky [1938, 1939] is a diminution of protein catabolism. This action on protein metabolism, moreover, is in Mirsky's opinion mediated through the pancreas. The hypertrophy of the pancreatic islets here observed may consequently be regarded not only as a manifestation of the pancreotropic action of the extract [Ogilvie, 1944], but also as part of the mechanism whereby the material reduces protein catabolism. This interpretation is supported by the knowledge that a similar growth of pancreatic islet tissue in the rat is accompanied by an equivalent increase in the amount of extractable insulin [Marks & Young, 1940], and that insulin exerts a definite nitrogen-sparing action in the dog [Mirsky, 1938]. Now another effect of the additional insulin is naturally an increase in the anabolic processes controlled by that secretion, and the carbon and nitrogen which were conserved as a product of the diminished metabolic rate are consequently synthesized respectively into fat [Rony, 1940] and protein [Mirsky, 1938, 1939] with a resultant increase in body weight. The transitory glycosuria which constantly accompanied this rise in body weight is explained by a temporary excess of the diabetogenic action of the extract over pancreatic islet activity, but the already noted increase of the islets in size and functional capacity induced by the pancreotropic property of the extract always ensued to neutralize the diabetogenic effect and cause subsidence of the condition. The fact that the glycosuria sometimes varied inversely as the body weight agrees with the observation of Young [1942] in the dog and was probably due to the loss of carbon and nitrogen incurred by the diabetes. Briefly, the reduced metabolic rate brought about by anterior pituitary extract can thus be ascribed to a combination of its diabetogenic and pancreotropic properties, while its pancreotropic influence is also responsible for the increase in anabolism and body weight. The rise in body weight, in other words, may be regarded as due to the diabetogenic activity of the extract balanced by increased pancreatic islet function induced through the pancreotropic action of the extract. Relatively excessive diabetogenic action or similarly decreased islet function, on the other hand, results in diabetes and ultimately in a loss of weight.

Such experimental results throw suggestive light on the genesis of human diabetes mellitus. As initially stated, the children who develop diabetes are often abnormally tall, while the majority of adult diabetic cases are or have been obese. Obese subjects at the same time do not increase in weight continuously, but acquire most of their overweight in the first few years and thereafter maintain a state of more or less equilibrium [Dunlop & Murray-Lyon, 1931]. They finally lose weight with the onset of diabetes. Further, Ogilvie [1935], assuming sugar tolerance to be an index of pancreatic islet activity, believes that the islets in a proportion of obese diabetic subjects pass through phases of increased, normal, and decreased function, while the fact that the islets in a considerable percentage of obese subjects are compensatorily hypertrophied [Ogilvie, 1933, 1935] also suggests that these structures are

overactive at first and later depressed. Finally, Rabinowitch [1938], having observed that diabetic subjects on caloric intakes definitely below theoretical requirements either maintain their weight or lose very much less weight than the anticipated amount, has thereby shown that the diabetic condition is characterized by a reduced metabolic rate, even although the basal metabolic rate is admittedly normal. Now all these phenomena—*increase and decrease in body weight, parallel phases of pancreatic islet function, hypertrophy of the pancreatic islets and reduction in the metabolic rate*—also obtained in the present pituitary-treated rabbits, and a mechanism similar to that described in these animals is consequently the logical one to assume for their correlation in the human diabetic subject. In other words, the pre-diabetic increase of height and weight in children and adults respectively, as Young [1941] has stated, may be regarded as due to excessive anterior pituitary activity, of which the diabetogenic action is temporarily compensated by the pancreatic islets, increased function of which is induced through the pancreotropic influence of the gland. Failure of the balance of this mechanism through islet exhaustion would ultimately result in diabetes mellitus.

#### SUMMARY AND CONCLUSIONS

1. Fifteen English rabbits maintaining an almost constant body weight and urinary volume on a practically fixed caloric intake were intensively treated with crude ox anterior pituitary extract.

2. The animals as a result of this treatment increased actually or relatively in weight on a definitely reduced caloric intake. The diminution in food value was due mainly to loss of appetite, but also partly to dissipation of energy through temporary glycosuria and ketonuria.

3. The fact that the injected rabbits relatively to their caloric intake excreted an excessive urinary volume leads one to infer that fluid retention played no part in their actual or relative increase in body weight.

4. The pancreatic islets of the treated animals, while numerically normal, were on the average more than twice as heavy as those of a control series.

5. The actual or relative increase in body weight of the injected rabbits on a reduced food value indicates that anterior pituitary extract reduces the metabolic rate and probably also increases anabolism. These effects are attributed to the diabetogenic action of the extract on the one hand and on the other to increased pancreatic islet function induced through the pancreotropic property of the preparation.

6. The above observations support the suggestion of Young [1941] that the pre-diabetic excess of height and weight in children and adults respectively is due to an elevated hypophysial-pancreatic balance, failure of which through islet exhaustion results in diabetes mellitus.

I wish to thank Prof. A. M. Drennan and Prof. F. G. Young for advice and criticism; Dr A. C. Aitken for a statistical analysis of the results relating to the islet tissue; Mr Ewart, manager of the Edinburgh Corporation Abattoir, for a supply of ox pituitary glands; Mr T. Allison for the chemical estimations; and Mr T. C. Dodds for the photograph used in Fig. 3.

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# EXPERIMENTAL CONTROL BY HORMONE ACTION OF THE OESTROUS CYCLE IN THE FERRET

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*(Received 10 October 1944)*

The changes that occur in the genital organs of the ferret during the oestrous cycle have been described by Marshall [1904, 1933], Robinson [1918], Hammond & Marshall [1930], Parkes [1930] and recently by Hamilton & Gould [1940]. In the present investigation here described our observations were mostly confined to the vulva which from being insignificant during the non-breeding season becomes enormous at oestrus, and is the most obvious indication that a ferret is on heat. Parkes [1930] and Hamilton & Gould [1940] have found that injected oestrogen can more readily induce cornification of the vagina than swelling of the vulva; moreover, it takes less oestrogen to produce changes in the uterus than in the vulva [Parkes, Rowlands & Brambell, 1932].

The ferret normally has two or three annual sexual seasons in the spring and summer, beginning usually early in April and lasting until September, autumn and winter being occupied by a prolonged anoestrous period during which ferrets never come on heat unless it is experimentally produced. The ferret does not ovulate unless it copulates, so that oestrus as manifested by the swollen vulva is often very prolonged, sometimes lasting for months. After ovulation the vulva almost immediately shrinks and during pregnancy or pseudo-pregnancy its size is like that in the anoestrous animal.

The experiments described in this paper were undertaken primarily to ascertain whether it was possible to maintain prolonged oestrus (as indicated mainly by the vulva) in previously anoestrous or spayed ferrets by oestrogen tablets and, if so, whether one could inhibit such an artificially produced oestrus by injections or tablets of progesterone. In the course of the experiments certain other questions of interest arose, and the bearing of our results on these is pointed out. It is to be regretted that war-time conditions limited the number of the experiments and the scope of the enquiry. Nevertheless, the results obtained are sufficiently clear, and by taking advantage of the great change in size which the vulva of the ferret undergoes they were far more striking than comparable results in most other mammals; the vulval changes can in fact only be likened to the swelling of the buttock and oedema of the sexual skin that develop as a consequence of oestrogen administration in monkeys [Zuckerman, van Wagenen & Gardiner, 1938].

Parkes & Bellerby [1926] showed long ago that the amounts of oestrogenic hormone required to induce oestrous changes in the vagina of the mouse were greater in pregnant animals than in spayed ones, and they supposed that this difference was due to the corpus luteum. They found also [1927] that luteal extract from the cow inhibited oestrus in the mouse, and Gley [1928] in a similar way found that luteal



extract from the sow inhibited oestrus in the rat. Robson (1937) and Courrier & Cohen-Solal [1937] found later that the cornifying effect of the oestrogenic hormones could be inhibited by the simultaneous administration of progesterone (cf. Robson [1936] for effects on the endometrium). Moreover, Robson [1938*a, b, c*] found that testosterone, which has a somewhat similar chemical constitution to progesterone, could also inhibit the effect of oestrogen (whether oestradiol or the synthetic substance triphenyl-ethylene). Further data on the antagonism between oestrogenic hormones and androgens has been published by Emmens & Parkes [1939], Emmens & Bradshaw [1939] and other investigators, injection experiments having been performed in each case.

Coming back to progesterone, Allen & Reynolds [1935] found that, whereas the rhythmic contraction of the rabbit's uterus is increased by oestrogen, this increase is inhibited by progesterone, which thus acts antagonistically. Zuckerman [1937] found that in injected monkeys there is a quantitative relation between oestrogen and progesterone on which the changes in the uterus depend and that an adequate amount of progesterone may counteract the influence of oestrogen.

Lastly, Burrows [1939] reported that progesterone appeared to reduce the gonadotrophin content of the pituitary, since mice which had received pituitary implants from progesterone-treated rats had smaller genital organs than those implanted with glands from normal controls.

It is obvious that the gradual and steady absorption from implanted hormone tablets simulates the natural conditions much more closely than the intermittent, uneven supply provided by injection methods. This method was introduced by Deanesly & Parkes [1937].

## EXPERIMENTS

### *Intact animals*

The results of fifteen experiments on intact ferrets are recorded below.

(1) A ferret, previously anoestrous, began to come on heat on 9 April 1941, and the vulva was fully swollen on 23 April. On 9 May the ferret was injected with 0.75 mg. of progesterone in 0.15 ml. of oil. No change in the vulva was detected. On 22 May the animal was injected with 5 mg. of progesterone in 1 ml. of oil. On the 26th the vulva appeared to be very slightly smaller but the effect was transitory. On 6 June five tablets of progesterone having a total weight of 121.5 mg. were implanted subcutaneously on the right side. On the 10th the vulva began to decrease in size and this continued daily until the 19th when the ferret was killed. The genital organs were preserved. The vulva was very much reduced. Sections of the ovaries showed numerous interstitial cells and many follicles but no ruptured follicles. The uterus was congested. The mammary glands showed some growth. The weight of the tablets recovered was 105.5 mg., showing that 16 mg. had been absorbed or an average of 1.23 mg. daily for 13 days.

(2) A ferret, which began to come on heat on 2 April, was injected with oestradiol (0.1 mg. in 0.1 ml. of oil) when it was fully on heat on 22 May to see if there was any apparent change in the mammary glands. The animal remained on heat for three months but no noticeable effect on the mammary glands could be observed and no other result was recorded.

(3) An oestradiol tablet weighing 10.3 mg. was implanted subcutaneously on the left side on 12 November. The ferret began to come on heat on the 17th and the vulva increased in size but did not reach the full oestrous dimension. Another tablet of oestradiol weighing 11 mg. was then inserted behind the old one. The ferret died apparently from internal sepsis affecting the left uterine cornu on 27 December. It was found that 2.7 mg. had been absorbed from the first tablet and 1.0 mg. from the second.

(4) This ferret was at first kept as a control. It was anoestrous in November and through the winter and began to come on heat on 15 April at the normal time. It remained continuously on heat until 29 August when the vulva began to subside. It was anoestrous on 9 September and remained so. On 14 December six tablets of stilboestrol dipalmitate weighing 83.7 mg. were implanted. It began to come on heat on the 17th and the vulva was much swollen on the 23rd. On 6 January four tablets of progesterone weighing 86 mg. were implanted and two days later the vulva was reduced, but the ferret died on the 9th. The uterus was very large and flaccid and very septic. 81.1 mg. of the progesterone were recovered but the oestrogen tablets were accidentally lost.

(5) The ferret began to come on heat on 15 April at the usual time. On 30 May four tablets of progesterone weighing 75.6 mg. were implanted on the right side. The animal began to go off heat on 3 June and the vulva thereafter gradually subsided. The tablets were removed on 17 June (weight 59.6 mg.), the amount absorbed having been 16 mg. On the 20th the ferret began to come on heat and was fully oestrous on 7 July. It then remained on heat until about 26 August and was anoestrous by about 9 September and continued so in the normal way until 24 March of the next year. It then came on heat again and the vulva remained swollen until it died. On 9 July 100 i.u. of chorionic gonadotrophin were injected into the jugular vein. The ferret died on 13 July. It was found after death that the uterus was extremely distended and full of pus.

On 24 July (the previous year) when this ferret was in full oestrus (after progesterone treatment the previous month, as recorded above) the mammary glands were markedly developed and the teats large; some serous fluid could be expressed. The hair on the mammary area was very thin. The mammary activity continued for some weeks and on 17 August it was noted that the fluid was milky. Later, when the vulva was subsiding the fluid became watery again and reduced in amount and by 7 October the mammary regression was complete.

(6) An anoestrous ferret had a tablet of stilboestrol dipropionate weighing 14.9 mg. implanted in its side on 14 December. The vulva began to enlarge on the 17th but never quite reached full normal oestrous size. The tablet was removed on 6 January and was found to weigh 12.5 mg., 2.4 mg. having been absorbed. The vulva was still much enlarged on that day, but was reduced on 8 January. The animal died from an unknown cause on 12 January.

(7) Two tablets of stilboestrol dipalmitate weighing 29.6 mg. were implanted into the right side of an anoestrous ferret on 14 December. The ferret began to come on heat as shown by the swelling of the vulva on 17 December and was fully oestrous about 6 January. On that day four tablets of progesterone weighing 81.1 mg. were inserted into the left flank. On 8 January the vulva was reduced and the ferret

became daily more and more anoestrous but not completely so, since the vulva remained very slightly swollen. On 22 January the progesterone tablets were removed when they weighed 60.0 mg. showing that 21 mg. had been absorbed. On 25 January the vulva began to swell again and was enlarged to full oestrous size on the 27th. The ferret remained on heat until it died on 12 March, when it was found that 1.6 mg. of the dipalmitate had been absorbed. The uterus was found to be much distended and the ovaries contained a number of small developing follicles.

(8) One tablet of stilboestrol dipropionate weighing 14.6 mg. was implanted in the right flank of an anoestrous ferret on 8 January. The animal began to come on heat as shown by the swelling of the vulva on 15 January. The vulva remained partially swollen on the succeeding days. On 22 January one tablet of progesterone weighing 17.1 mg. was implanted into the left flank. On the 25th the vulva had become reduced to anoestrous size. The tablets were removed on 1 February. The stilboestrol dipropionate then weighed 13.2 mg., showing that 1.4 mg. had been absorbed, and the progesterone weighed 16.3 mg. showing that 0.8 mg. had been absorbed. The animal died on 8 February when the uterus was found to be much distended and septic.

(9) One tablet of stilboestrol dipalmitate weighing 14.1 mg. was implanted into the right flank of an anoestrous ferret on 8 January. On 15 January the vulva began to swell and after some fluctuation in size was fully oestrous on 10 March and remained so until 21 May. It copulated on 3 May. The prolonged oestrus occurring in the winter is possibly to be regarded as a summation effect of the hormone from the ferret's own ovaries and the low dose of stilboestrol dipalmitate absorbed from the tablets. It was almost anoestrous on 27 May and completely so on 9 June. It had fully moulted by 11 June. It did not apparently become pregnant but the vulva remained of anoestrous size until 6 July. Moreover, the mammary glands were thickened on 17 June and were more so on the 21st. The vulva again began to swell on 6 July and the ferret was fully on heat from 14 July until 1 September; it then subsided and the animal was continuously anoestrous until 1 December. At that time it came on heat again and remained on heat until it died on 30 March. It is suggested that the winter oestrus was due to a greater degree of absorption from the tablet at this time. The weight of the tablet recovered was 11.3 mg., showing that 2.8 mg. had been absorbed. Post-mortem examination showed an enormously enlarged spleen, weighing 10.97 g. The liver was pale. The uterus was distended and full of pus.

(10) Two tablets of stilboestrol dipalmitate, weighing 28.5 mg. were implanted into the right flank of an anoestrous ferret on 8 January. The ferret began to come on heat on the 13th. The vulva swelled but did not become fully enlarged to normal oestrous size until 3 February. The ferret then remained continuously on heat until 3 May. It copulated on 24 April. The vulva began to diminish on 4 May and was reduced to anoestrous size on the 21st. The ferret remained apparently anoestrous until 4 June when the vulva again began to swell. The ferret was moulting on 11 June. It showed definite thickening of the mammary glands on the 17th. It was fully on heat on the 24th but died on 2 July. Post-mortem examination did not reveal any swelling of the uterus but rather anaemia which had apparently affected the other organs. The weight of the recovered tablets was 26.8 mg., showing that 1.7 mg. had been absorbed.

This ferret and the two preceding ones (nos. 8 and 9) were virgins and litter-mates, as was also the next animal (no. 11).

(11) This ferret was a control to the three preceding animals. It came on heat at the usual time in April and remained continuously so until 25 June. It copulated on 18 June. A laparotomy on 2 July showed that it was not pregnant. Nevertheless, the vulva became diminished in size on 25 June and remained so until 6 July. The vulva then swelled again intermittently until 19 July. The ferret was moulting on 16 July. The vulva was apparently completely anoestrous from 22 July to 21 August when the ferret again began to come on heat. It was fully oestrous from 25 August to 4 September, when it again became anoestrous and remained so through the autumn and winter. It came on heat again about 10 April and was continuously oestrous until 24 June. It was injected subcutaneously with 100 i.u. of chorionic gonadotrophin on 16 June. It was fully anoestrous from about 6 July until 11 August, since which time it was again oestrous until September. It had completely moulted by 22 July. That ovulation was induced and that a pseudo-pregnancy followed may be concluded from the length of the anoestrous period and from the moulting. Three untreated animals in this year moulted in the order in which they were mated up and before any of the others (an unmated control, two implanted castrates, and three intact animals with stilboestrol implants) had shed their coats.

(12) A ferret which was on heat when it was obtained in May was continuously oestrous until 9 July. It was then injected with 100 i.u. of chorionic gonadotrophin. The vulva began to be reduced on the 12th and became further so on the succeeding days. On the 23rd one tablet of stilboestrol dipropionate weighing 14.4 mg. was implanted on the left side. On the 26th the vulva was nearly fully swollen and the ferret was again oestrous over the next few days. The vulva was slightly reduced again for the first week in August, but the ferret died on the 9th. The kidneys were found to be much enlarged but there was no apparent sepsis in the uterus or elsewhere. The recovered tablet weighed 13 mg., indicating an absorption of 1.4 mg.

(13) Two stilboestrol dipalmitate tablets weighing 26.8 mg. were inserted into the left flank of an anoestrous ferret on 4 January. The vulva showed some slight signs of swelling on the 14th but they only persisted a few days, anoestrous size having been resumed on the 31st. The vulva did not begin to swell again until 4 April, the time for the normal breeding season. The ferret was fully on heat from 17 April until it died on 20 July. The uterus was enlarged and contained a clear fluid. The left kidney had a small septic area. The ferret had been having diarrhoea. The weight of the recovered tablets was 26.4 mg., showing that 0.4 mg. had been absorbed. The ferret copulated on 17 July.

(14) One stilboestrol dipalmitate tablet weighing 14.6 mg. was inserted into the left flank of an anoestrous ferret on 4 January. The vulva began to swell on the 7th and the ferret was continuously oestrous with occasional slight fluctuations in the size of the vulva until it died on 28 July just after it had started to moult. There was a large blood clot in the bladder and several large calculi. The uterus was of normal size. The tablet was found but unfortunately broke up so that the amount of absorption was unknown.

(15) One stilboestrol dipalmitate tablet weighing 14.5 mg. was inserted into the left flank of an anoestrous ferret on 4 January. The vulva began to swell on the

10th and was about half the normal oestrous size on the 31st. On 7 February the vulva was slightly less and became further reduced, being completely anoestrous from 4 March to 19 April. The ferret then came on heat and remained so until it died on 30 August. Latterly the vulva was extremely enlarged and showed some haemorrhage. Post-mortem examination showed advanced pulmonary disease. The uterus and other generative organs were of normal size. The tablet was recovered and weighed 13.4 mg. showing that 1.1 mg. had been absorbed. The ferret was moulting on 22 July.

#### *Oophorectomized and hysterectomized animals*

In the experiments which follow the animals were operated upon.

(16) The ovaries were removed from an anoestrous ferret on 27 October. The ferret had been on heat normally through most of the summer and until 24 September. On the same day as the operation one oestradiol tablet weighing 9 mg. was implanted subcutaneously on the left side. The vulva swelled on 29 October and was fully swollen on 5 November. On 12 November five progesterone tablets weighing 94.2 mg. were implanted on the right side but these became extruded and lost and the vulva remained swollen. On 24 November five more progesterone tablets weighing 78.8 mg. were implanted on the right side. Soon after the animal became ill. The vulva became slightly reduced. The ferret died on 3 December. The uterus was large and flaccid, but not congested. The weight of the progesterone tablets recovered was 70.9 mg., showing that 7.9 mg. were absorbed. The other tablets could not be found. The animal was very fat.

(17) An oestrous ferret copulated on 7 May but the vulva on the succeeding days did not diminish in size. On 14 May the ovaries and uterus were removed when the vulva was still large and oestrous. The vulva began to diminish on the 15th and was fully reduced to anoestrous size on the 21st. On 12 June two tablets of stilboestrol dipalmitate weighing 26.3 mg. were implanted on the left side. On the 14th the vulva began to swell and on the 17th was fully swollen. On 22 June four tablets of progesterone weighing 82.9 mg. were implanted on the right side. The vulva was diminished on 25 June and became further reduced. On 19 July it was completely anoestrous in size. On 4 August the progesterone tablets were removed; they weighed 48.9 mg. showing that 34 mg. were absorbed. The vulva was about half the oestrous size on 6 August and full oestrous size on the 9th. It remained so until the ferret died on 25 August. The liver was apparently infected. The stilboestrol tablets recovered weighed 25.4 mg. showing that 0.9 mg. were absorbed.

(18) An oestrous ferret copulated on 18 May but the vulva did not consequently diminish. On the 24th the ovaries and uterus were removed. The vulva was nearly anoestrous on the 29th and completely so on 11 June. One tablet of stilboestrol dipropionate weighing 14.5 mg. was implanted on the left side on 12 June. The vulva began to enlarge again on 14 June and was fully enlarged on 16 June. Four tablets of progesterone weighing 70.8 mg. were implanted on the right side on 22 June. The vulva began to diminish on the 24th; it was nearly anoestrous on 12 July but never became completely so. On 23 July one progesterone tablet which was protruding from the open wound was removed and the others were probably extruded. The tablet removed weighed 8.1 mg. On 16 August each side was opened at the site

of tablet implantation but no tablets could be found. Two tablets of stilboestrol dipalmitate weighing 26.8 mg. were then implanted on the left side posteriorly. The vulva which had never become completely reduced in the previous weeks then became enlarged to full oestrous size on 18 August. Two tablets of testosterone weighing 101 mg. were implanted on the right side over the ribs on 7 September, and another tablet weighing 48.1 mg. on 6 October. The vulva was slightly softened on 13 September and remained very slightly reduced until 1 October when it again attained full oestrous size. The second implantation of testosterone was followed after 2 days by a further softening of the vulva but the change was very slight and it cannot be claimed for certain that the testosterone produced any result. On 13 October the first two testosterone tablets were found to be protruding; the ferret was anaesthetized and died under the anaesthetic. The weight of the first two testosterone tablets found was 50 mg. showing that 51 mg. had been absorbed. The weight of the additional tablet was 48.1 mg. or the same weight as when it was implanted. No trace of any of the internal generative organs could be found.

(19) On 7 September the ovaries and uterus were removed from a ferret which had been on heat during the summer over a long period, but at that date had begun to go off heat. On the same day two tablets of stilboestrol dipalmitate weighing 25.4 mg. were implanted on the left side. The vulva which was then about half oestrous size became fully oestrous by 11 September and remained so. On 6 October four tablets of progesterone weighing 58.9 mg. were implanted on the right side. On the 9th the vulva began to subside and was almost completely anoestrous on the 20th. It remained so until after the progesterone was removed, this being done on the 27th. The weight of the tablets was 46.8 mg. showing that 12.1 mg. had been absorbed. On 1 November the vulva was almost fully oestrous and completely enlarged on the 3rd. The vulva was fully enlarged until the ferret died on 20 January. The stilboestrol tablets recovered weighed 24.4 mg., showing that about 1 mg. was absorbed. On 10 November six testosterone tablets weighing 300.8 mg. were implanted and on the 25th six more weighing 296.3 mg. were implanted. On 11 December all the testosterone tablets were removed; the first six weighed 143.6 mg. and the second six weighed 231.5 mg., showing that 157.2 mg. and 64.8 mg. were respectively absorbed. After the implantation of the testosterone the vulva showed no decrease in size but the surface became dry from about 13 November onwards and after their removal it became much more moist and turgid.

(20) A ferret which had been anoestrous from August onwards was injected with 50 i.u. of pregnant mare's serum gonadotrophin on 17 October, with 100 i.u. on the 20th, with 100 i.u. on the 23rd and with 200 i.u. of chorionic gonadotrophin on the 27th. The vulva began to swell on the 22nd and was nearly full oestrous size by the 29th. On that day the ovaries and cornua uteri were removed. The vulva was almost completely reduced on 1 November and entirely anoestrous by 5 November. Two tablets of stilboestrol dipalmitate weighing 14.4 mg. and 14.7 mg. were implanted into the dorsal part of the loin on 10 November. The vulva began to swell on the 13th and was fully oestrous on the 24th remaining so until 7 January when it began to get smaller. The ferret was unwell about this time but fully recovered in March. The vulva fluctuated in size and was completely anoestrous on 4 February, but

started to swell again on 11 March and was fully oestrous on 13 April, remaining so until its death. It copulated on 31 May for about two hours. After copulation the fluid on the vulva was examined and found to contain very numerous living spermatozoa. The subsequent size of the vulva was not affected by copulation. The ferret died on 22 July when the alimentary canal was found to be locally congested and to have adhesions. A trace of one uterine horn was found but otherwise the internal generative organs were removed. The two stilboestrol tablets were recovered and weighed 27.3 mg., showing that 1.8 mg. had been absorbed.

(21) The ovaries and Fallopian tubes but not the uterus were removed from an anoestrous virgin ferret on 14 December. Two tablets of stilboestrol dipalmitate weighing 28.9 mg. were implanted on the left side on 26 January. The vulva began to swell on the 28th, was nearly full oestrous size on 7 February and completely so on the 14th. In the meantime on 11 February four tablets of progesterone weighing 59.9 mg. were implanted on the right side. The vulva was very much reduced on the 21st and completely anoestrous on the 28th. On 1 March the progesterone tablets were removed and found to weigh 47.8 mg. showing that 12.1 mg. had been absorbed. On 4 March the vulva was again swollen and was almost fully oestrous from 6 March until 20 March. From 8 March to the 17th the ferret was injected daily with 25 mg. of testosterone propionate, from the 18th to 21st it was injected daily with 50 mg. of the same, and on the 22nd and 23rd it was injected with 40 mg. of free testosterone. From 20 March until some time afterwards the vulva fluctuated in size, but was never fully swollen or completely reduced until 25 May when it became entirely anoestrous. On 7 June and without having any further treatment since March the vulva became swollen again and with some variation remained about half so until 17 July. It subsequently swelled further but not so as to be completely oestrous. It became reduced on 28 July and was almost completely so on 2 August. It started to swell again on the 11th and was fully oestrous on the 14th, remaining so until the present date (17 November). Variations in the vulva are difficult to explain, but the resumption and continuation of complete oestrus late in the season when ferrets are not normally on heat was clearly due to the stilboestrol tablets. The ferret was moulting on 22 July.

#### REMARKS

In the above experiments the amount absorbed from the implanted tablets is given as the difference between the dry weights before implantation and those after removal. Deposition of protein within tablets would make the amount given rather too low in the case of the stilboestrol dipropionate tablets and the testosterone given to the ferret in Exp. 18. The oestradiol and progesterone tablets had been previously implanted into other animals; the testosterone given to the ferret in Exp. 19 was in the form of fused pellets; the stilboestrol dipalmitate tablets were also very compact. Absorption from these last is so slight that the error in cleansing and weighing becomes appreciable. Figures are given in individual cases. The mean amount absorbed per tablet per day was 4.4  $\mu$ g. equivalent to 1.6  $\mu$ g. of free stilboestrol.

The experiments show clearly enough that implanted tablets containing a potent oestrogen will produce and maintain oestrus in ferrets deprived of their ovaries or

previously anoestrous as in midwinter, thus confirming the results of injection experiments on other animals by various investigators. It is shown further that progesterone tablets introduced into such animals will inhibit the oestrous condition, the oestrus being resumed upon the removal of the progesterone; this also accords with and extends the conclusions of other observers. It is obvious also that it confirms the view that the corpus luteum in other animals inhibits oestrus during the dioestrus. It will be seen that there is some evidence that a smaller quantity of introduced oestrogen may cause heat in intact animals than in ferrets which have had their ovaries removed, the presumption being that the ovaries of a normal animal are always secreting oestrogen even in the anoestrous season, though in small quantities, and that heat induced in such animals as a result of a small amount of additional hormone from an implanted tablet is a summation effect. Moreover, in Exps. 9 and 10, in which the ferrets were intact and had implants, the vulva subsided after copulation (but not in the oophorectomized ferret in Exp. 20) and remained reduced for some time as though they were pseudo-pregnant. It seems likely, therefore, that these ferrets ovulated and hence must have had large follicles when mated. There is some evidence that testosterone, which has a chemical constitution like that of progesterone, may have a similar inhibitory effect upon oestrus. It is probable that different individuals have different capacities to respond to oestrogen, but it must always be remembered that the rates of absorption may vary according to the precise position of the implanted tablets. Moreover, it is shown definitely that even oophorectomized ferrets can vary in their response at different times, since induced oestrus may temporarily cease and later be resumed without any further operation or introduction of tablets. Also, the vulva may sometimes subside if the ferret is unwell. A further result of tablet implantation which was more apparent in some individuals than in others was the development of the mammary glands and their secretion of milk or of a milky substance.

It is likely that a condition of oestrus extended over a long time as a result of implanted oestrogen may decrease the power of resistance to disease, since many of the ferrets so treated developed a septic condition of the uterus and sometimes of other organs.

#### SUMMARY

1. Tablets of oestrogen (either oestradiol or a diethyl-stilboestrol ester) implanted into anoestrous or oophorectomized ferrets will produce and maintain oestrus for an indefinite period.
2. Progesterone tablets implanted into ferrets with experimentally induced oestrus will inhibit such oestrus and on their removal oestrus will be resumed. Thus progesterone antagonizes oestrogen.
3. There is some evidence that intact anoestrous ferrets may be brought on heat by less implanted oestrogen than that which is required to induce heat in oophorectomized ferrets.
4. There is some evidence that testosterone may antagonize oestrogen in the same way as progesterone does.
5. The capacity for response to introduced oestrogen may vary in different ferrets and at different times in the same ferret.



6. Two 15 mg. stilboestrol dipalmitate tablets were found regularly to be sufficient to cause swelling of the vulva; the mean daily absorption per tablet was  $4.4 \mu\text{g.}$ , equivalent to  $1.6 \mu\text{g.}$  of free stilboestrol.

We have to thank Messrs Organon for testosterone and its propionate. One of us (J. H.) had a research grant from the Agricultural Research Council.

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# INDUCED OVULATION AND HEAT IN ANOESTROUS SHEEP

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(Received 10 October 1944)

If hormone treatment is to be applied commercially on a large scale to control the breeding season, the products used should be cheap and readily available. At the present time such substances are limited to serum gonadotrophin (pregnant mares' serum), and to stilboestrol and other synthetic oestrogens. The investigation here reported concerns the possibility of obtaining fertility in sheep by a single treatment with serum gonadotrophin and stilboestrol. Hammond, Hammond & Parkes [1942] have reviewed much of the work in this field; it is convenient to summarize this briefly, referring to particular findings in connexion with the results as they are presented.

Oestrogens will generally induce heat in anoestrous sheep, and will quite often cause ovulation—presumably by stimulating release of ovulating hormone from the pituitary of the treated animal. Serum gonadotrophin and other follicle-stimulating preparations will consistently bring about ovulation; it seems likely that the animal's pituitary is here also involved—activated by oestrogen from the stimulated ovaries. The ovulation induced by serum gonadotrophin, like the first ovulation of the natural breeding season, is not normally accompanied by heat. An induced ovulation is sometimes followed, a cycle interval later, by spontaneous ovulation and heat. In the presence of an active corpus luteum, serum gonadotrophin (and oestrogen) may cause ovulation, but generally ovulation is inhibited until the activity of the corpus luteum wanes. By giving two serum gonadotrophin treatments about 16 days apart ovulation, accompanied by heat, can be induced; much, however, remains to be determined as to optimum dosage and interval between injections, and it appears also that nutritional conditions greatly affect the proportion of animals coming on heat. The number of ovulations induced by serum gonadotrophin during anoestrus is normal and unrelated to the dose of gonadotrophin; when a corpus luteum is present, it is related to both dose and interval between injection and waning of the luteal activity. Similar findings have been reported for the cow [Hammond & Bhattacharya, 1944].

## MATERIALS AND METHODS

*Sheep.* With the exception of three Ryeland hoggets (nos. 188–190) which appeared to have been mated in their first season and to have lost their lambs, the ninety animals used were all Suffolks, or Suffolk or Hampshire crossed with the Border Leicester-Cheviot. The ewes were all without any apparent anatomical cause for sterility: some were crone ewes, the remainder had probably aborted or had lost their lambs. The hoggets were late-born animals; only about half of them had ovulated in the previous breeding season. The lambs were early born and well grown. All of them had been sent to the Cambridge collecting centre for slaughter.

These animals are similar to those used by Hammond *et al.* [1942]. An account of the normal anoestrous reproductive state of hoggets such as these has been published [Hammond, 1944]; active corpora lutea are to be found in about 5% of them and about half have fair-sized follicles. It might be expected that lambs would show less ovarian activity, and on that account be unsuitable material for work to be applied to adult ewes. However, we have no information on the ovarian state of lactating ewes; since lactation can delay onset of the breeding season, it is not unlikely that they show less activity than hoggets. One of the lambs killed was found to have ovulated spontaneously.

*Substances used. PMS.* A single batch of water-soluble dried powder, assayed at 75 i.u./mg., was used. Solutions in distilled water were made up freshly before injection. Treated animals all received 750 i.u. subcutaneously in a volume of 1-2 ml.

*Progesterone.* Progestin B.D.H., 5 units per ml. of oil, was injected subcutaneously.

*Stilboestrol di-n-butyrate* was injected subcutaneously in arachis oil, in such concentration that each injection was in a volume of 1 ml. Dosage is expressed as weight of dibutyrate (for correction to free stilboestrol,  $\times 0.66$ ).

*Stilboestrol.* (a) In arachis oil solution (1 mg./ml.) given subcutaneously. (b) In arachis oil emulsions (2.5 mg./ml.) given intramuscularly or intravenously. The emulsion contained 20% oil and 5% lecithin added to 1% sodium carbonate solution, and was finally brought to about pH 8.5 by addition of a few drops of conc. HCl. (c) In suspension in normal saline plus 0.7% lecithin (1.5-2.5 mg./ml.) given intramuscularly or intravenously; prepared by dissolving stilboestrol and lecithin in ether, adding to the saline and removing the ether under reduced pressure.

Subcutaneous injections were given in the axilla; on different sides when there was more than one. Intravenous injections were given into the jugular vein, intramuscular injections deep in the thigh, with not more than 3 ml. in one leg.

*Methods.* Because ewe lambs, though not on heat, may sometimes stand for the ram until he begins to mount, and because an old ram will try ewes more thoroughly than a young one, a 6-year-old ram was run with the first lot of sheep. None were served and it was thought wise to replace him by a younger ram, as the older one did not seem very keen. This younger ram was used for the rest of the experiment; he was sexually active throughout the period and was producing good quality sperm at the time first used. He was not periodically checked. A third ram was used later, five of a group being run with this ram, the other five with the younger one. He had been used for sperm collection (to which he is well trained) the previous week; as none of those with him were served, while two of the five others were, his activity must yet be considered doubtful.

The sheep were received in groups of ten weekly, and were all killed seven days afterwards—nos. 121-130 on 25 May, nos. 201-210, the last group, on 17 July. They were run, until within an hour of slaughter, with an ochred ram. Injections were given at about the same hour as that of killing, the sheep were inspected daily at this hour for service, and, in groups in which oestrogen was given to some after serum gonadotrophin, 12-hourly after that time. Onset of oestrus came within the time stated. Animals treated only with serum gonadotrophin were inseminated, partly in the hope that the condition of the ova recovered would indirectly indicate the time of ovulation. The sperm used was freshly collected and all of good quality.

At the slaughterhouse, the following particulars were recorded: number of fresh ovulations, number of follicles classified according to size, and character and amount

of vaginal mucus. Ovaries and uteri were then removed to the laboratory in a damp towel, where they were weighed, and the tubes and upper halves of the uterine cornua corresponding to fresh corpora lutea were flushed with normal saline in search of ova. Apart from determining whether ova were fertilized, the object of searching for ova was to see if the position of ova in tube or uterus agreed with the time of ovulation as indicated by the appearance of the corpus luteum, or if the rate of tubal transport of ova was affected. Efficiency of recovery was not very great—it was certainly not to be expected in the case of uterine washings; in the absence of control washings after normal ovulation there is no good reason to attribute it to the 'entrapment' suggested by Murphree, Warwick, Casida & McShan [1944] in the case of induced multiple ovulations.

## RESULTS

### *General*

Details of the animals, except of those found to have ovulated before treatment, are given in Tables 1–4, where they are listed according to the treatment given. In each group of ten animals an attempt was made to compare the effects of the different treatments, the animals being paired off as nearly as possible. From its supposed action on the pituitary it was to be expected that stilboestrol, given at the same time as serum gonadotrophin, would inhibit the ovulation produced by serum gonadotrophin. It was therefore attempted to obtain heat by giving oestrogen a day later than the serum gonadotrophin, and to induce it sufficiently early for fertilization to be possible. Having failed with various doses and methods of administration of stilboestrol, the effect of preliminary treatment with small amounts of stilboestrol dibutyrate was investigated; this also showed little promise of leading to a practical method of obtaining fertility in anoestrous sheep.

### *Sheep with active corpora lutea when injected*

There were seven of these (124 and 134 were wethers included by accident) which may be briefly mentioned. One only came on heat; this was 156, treated with 5 mg. of stilboestrol one day after serum gonadotrophin and 3 days before killing. It was on heat 24 hr. before killing, at which time the corpus luteum had regressed and there were seven large follicles present in the ovaries. In 173 the corpus luteum had also regressed; serum gonadotrophin had been given 5 days before killing, and 10 mg. of stilboestrol a day later; there had been a single ovulation, the ovum being still in the tube; heat may in fact have occurred as this animal was with a ram of doubtful activity.

Ewe 132 was 10 weeks pregnant, received serum gonadotrophin and 5 mg. of progesterone 5 days before killing; it had four corpora lutea of pregnancy, a single foetus, and two fresh ovulations. Ewe 145 was 18 weeks pregnant, received serum gonadotrophin and 4 mg. of stilboestrol 4 days before killing; it had two corpora lutea of pregnancy, a single lamb, and no fresh ovulations or any follicles of any size.

Hogget 133 received serum gonadotrophin and 1 mg. of stilboestrol 5 days before killing; it ovulated four times. Hogget 166, with the same dose of serum gonadotrophin 4 days before death and 5 mg. of stilboestrol a day later, ovulated twice. Lamb 169 given 4 mg. of stilboestrol intramuscularly in suspension 4 days before

Table 1. *Sheep injected with 750 i.u. of mare serum gonadotrophin alone, or with progesterone*

No.	...	122	123	129	130	137	138	141	142	143	158	159	167	168	198	199	200	131*	160*
Ewe, hogget or lamb	L	L	H	H	H	L	H	E	E	E	L	H	L	H	L	L	L	E	H
Time inseminated after injection (days)	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	0	.	.
Injection to killing (days)	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	5	4
Fresh corpora lutea	3	2	1	2	3	2	1	1	1	2+4	2	1	3	1	1	2	3	2	3
Ova recovered: Tubes	.	.	.	1	.	.	.	.	1	2	2	1	3	1	.	2	.	3	3
Uterus	N.W.	N.W.	N.W.	N.W.	N.W.	N.W.	N.W.	.	.	.	.	.	.	.	.	.	1	2	.
Follicles: Large	1	.	1	1	3	.	1	3	.	2	.	.	.	1	.	1	.	1	.
Medium	1	.	1	1	1	2	2	1	2	.	.	2	2	.	.	1	1	.	.

\* 131 and 160 received 5 mg. of progesterone 1 day before the gonadotrophin, and 160 a further 5 mg. at the same time as the gonadotrophin. 131 was possibly served within 2 days of injection of gonadotrophin. None of the others came on heat.

N.W. Not washed.

Table 2. *Sheep injected with stilboestrol alone*

No.	...	121	125	126	127	139	140	147	149	150	157	170	191	192	201	202	209	210
Ewe, hogget or lamb	L	L	H	H	H	L	H	E	E	E	L	L	L	L	L	L	L	L
Substance	1	1	1	1	1	1	1	4	4	4	5	5	4	4	4	4	4	4
Dose (mg.)	†	†	†	†	†	†	2†	.	.	3	3	1½	.	2½	†	5	†	†
Injection to heat (days)	5	5	5	5	5	5	5	4	4	4	4	4	6½	6½	6½	6	6	6
Injection to killing (days)	.	.	.	.	.	.	1	2	.	2	.	.	1	.	.	1	1	2
Fresh corpora lutea	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.
Ova recovered: Tubes	.	.	.	†	†	.	.	1	.	2	.	.	.	.	.	.	.	.
Uterus	.	.	.	N.W.	N.W.	.	1	N.W.	.	.	.	.	1	.	.	1	.	.
Follicles: Large	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.
Medium	2	1	.	2	1	1	2	.	1	.	1	.	1	.	.	.	1	.

\* 201, 202, a second injection of the same amount given 1½ days later.

† Ram of doubtful activity.

‡ Tube blocked.

All injections subcutaneously in oil, except 170 (intravenously in suspension).

N.W. Not washed.

Table 3. *Sheep injected with 750 i.u. of mare serum gonadotrophin followed by free stilboestrol 24 hr. later*

Table 3. *Sheep injected with 150 i.u. of mare serum gonadotropin*

No....	164	165	101	102	103	104	105	171	172	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190
Ewe, hogget or lamb	H	H	L	H	L	L	L	L	L	L	H	E	E	H	E	L	L	L	L	L	L	L	L	H	H	H
Stilboestrol dose (mg.)	5	5	5	5	5	5	5	10	10	10	10	10	10	10	10	10	8	8	8	8	8	8	8	8	8	8
PMS injection to heat (days)	3	.	3	1½	2½	.	*	4	*	.	.	*	.	.	.	3	.	.	.	.	.	.	.	3½	.	5
PMS injection to killing (days)	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Fresh corpora lutea	4	1	2	3	2	1	1	1	1	.	2	2	2	1	2	.	1	1	1	1	2	2	2	1	2	1
Ova recovered: Tubes	4	1	1	2	1½	1	1	1	1	.	1½	.	1	1	1½	.	.	.	.	.	.	.	†	.	.	1
Uterus	.	.	N.W.	.	†	N.W.	.	.	.	3	1	.	.	.	N.W.	.	†	.	.	.	1	.	1	1	.	2
Follicles:	.	.	2	.	2	1	.	.	.	.	.	.	.	.	.	2	1	1	1	1	.	.	1	.	1	2
Large	.	.	.	.	.	.	4	.	.	.	1	3	3	.	.	.	.	.	1	.	.	.	1	.	.	1
Medium	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.

\* Ram of doubtful activity.

† Washings lost, or tube blocked.

Stilboestrol given in oil subcutaneously to 154 and 155; in oil emulsion to numbers 171-180, in suspension to 101-105 and 181-100—intravenously to 104, 105, 170, 178, 180, 181-184 and 188, intramuscularly to the remainder.

N.W. Not washed.

Table 4. *Sheep given 750 i.u. of mare serum gonadotrophin and free stilboestrol at the same time or 12 hr. later, and others given stilboestrol di-n-butylate before the gonadotrophin injection*

No. ...	128	135	136	144	146	148	161	162	163	103	104	105	106	107	203	204	205	206	207	208
Ewe, hogget or lamb	H	H	H	E	E	E	H	H	H	L	L	L	L	L	L	L	L	L	L	L
Stilboestrol } days before PMS	.	.	.	.	.	.	.	.	.	2½	2½	2½	2½	2½	2½	2½	2½*	2½	2½	2½
di-n-butylate } dose (mg.)	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Free } days after PMS	0	0	0	0	0	0	5	6	6	.	.	.	.	.	.	.	.	.	.	.
stilboestrol } dose (mg.)	1	1	1	4	4	4	5	6	6	.	.	.	.	.	.	.	.	.	.	.
PMS injection to heat (days)	†	.	.	2	3	.	.	.	3½	.	.	.	.	.	2	3½	3	3	.	.
PMS injection to killing (days)	5	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Fresh corpora lutea	2	1	2	.	.	.	2	1	2	1	1	.	1	1	.	2+2	.	.	1	.
Ova recovered: Tubes	×	1	.	.	.	.	1	2	.	2	1	.	.	.	.	2	.	.	.	.
Uterus	N.W.	.	1	.	.	N.W.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Follicles:	.	2	1	2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Large	1	.	.	.	3	.	.	1	2	.	.	.	.	.	.	.	.	.	.	.
Medium	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

\* A second injection of the same amount given 1½ days later.

† Ram of doubtful activity.

× Doubtful service; × washings lost.

All stilboestrol injections subcutaneous in oil, except 105-7 and 203-8, in which free stilboestrol was given intramuscularly in suspension.

N.W. Not washed.

killing had two large mid-cycle corpora lutea and no follicles of any size and no fresh ovulation.

These animals came to be grouped together entirely by chance and afford no very interesting comparison either of different treatments or with animals without corpora lutea when injected. The absence of follicles in the ewe toward the end of pregnancy (145) is perhaps worthy of remark.

### *Ovulation*

All animals treated with serum gonadotrophin were killed 4 or 5 days later, as were those given free stilboestrol alone; six animals treated with stilboestrol dibutyrate only were killed after 6 or 6½ days. All sixteen given serum gonadotrophin alone, and two given progesterone as well (Table 1) had ovulated. Those given serum gonadotrophin followed by stilboestrol 24 hr. later (Table 3), twenty-six in all, had ovulated with two exceptions (174, 180); these two had very large tense follicles, cystic in appearance. Three given stilboestrol 12 hr. after serum gonadotrophin had ovulated, two out of six in which stilboestrol and gonadotrophin were given together had not ovulated (Table 4). One of these (144) had one very large, tense and cystic-looking follicle. Inhibition by stilboestrol of ovulation produced by serum gonadotrophin has been reported by Frank & Appleby [1943].

Eleven animals (Table 4) were given a small dose of stilboestrol dibutyrate 2 days before the serum gonadotrophin was given, in nine of them free stilboestrol was given a day later. Five of these nine had not ovulated; in at least three (194, 196, 204) of the six which had ovulated, ovulation was attributable to the preliminary treatment with stilboestrol dibutyrate. 206, which had not ovulated, had a very large cystic-looking follicle.

Of those animals receiving stilboestrol alone (Table 2), seven out of eleven receiving 1–5 mg. of stilboestrol had not ovulated, while five out of six treated with ¼ or ½ mg. of di-*n*-butyrate had ovulated. These figures appear to suggest that a small amount of stilboestrol is more efficient in inducing ovulation. Consideration should however be given to those other animals similarly treated with dibutyrate, followed by gonadotrophin (Table 4); five of these eleven had not ovulated—although they had received serum gonadotrophin also. These five had been given free stilboestrol 3 days before killing—but 3 or 3½ days after the dibutyrate—at which time ovulation should have already occurred; for all ova recovered from animals treated with dibutyrate alone were in the uterus, and all their corpora lutea appeared at least 4 days old—as they did in some of those treated with both dibutyrate and gonadotrophin.

It thus seems very probable that stilboestrol induces ovulation only in sheep which have already a fair-sized follicle present in the ovary, whereas serum gonadotrophin will induce ovulation whether such a follicle is present or not; though this action may be inhibited by stilboestrol in cases where stilboestrol alone is ineffective. If this is true, it might be expected that, in those animals ovulating, the average interval between injection and ovulation would be greater for serum gonadotrophin than for stilboestrol, for about half of those ovulating after the former would have had first to develop a large follicle.

The material is inadequate for investigation of this point; the appearance of the corpora lutea does not give a sufficiently reliable indication of the time of ovulation

and estimates of this time must depend upon whether ova were in the tubes or the uterus, assuming that the rate of transport is normal and unaffected by the treatment given.

Both with stilboestrol or serum gonadotrophin given alone, ova recovered 4 days after injection were nearly all in the tubes, and in those killed 5 days after injection probably nearly all ova were in the uterus. In those given gonadotrophin and stilboestrol at the same time or later all ova recovered were in the tubes 4 days after the gonadotrophin injection; 5 days afterwards they were recovered from tubes in seven ewes and from the uterus in five, but allowance must be made for the smaller chance of recovery from the uterus. There is thus little indication that stilboestrol in the dosage employed affected the rate of transport of tubal ova, and it seems likely therefore that the rate is much the same as that after normal ovulation.

Kelly [1937] found unfertilized ova to enter the uterus about 70 hr. after the end of heat, and Clark [1934] that fertilized ova did so about 110 hr. after the beginning of heat. These two estimates seem to agree within about 12 hr. On this basis induced ovulations would have occurred between 24 and 60 hr., for the most part, after injection; probably the majority were between 24 and 48 hr.

The number of ovulations falls within the normal limits in all cases, except that a second ovulation occurred in two animals (143, 204), probably about 2 days after the first ovulation. Follicles with ovulation cones were seen in a few of the other sheep injected with serum gonadotrophin, alone or with stilboestrol, which had already ovulated. No such follicles were seen in animals treated with stilboestrol alone.

### *Heat*

None of the animals given gonadotrophin alone came on heat; one of two given progesterone was marked by the ram—but at a time when ovulation may have already occurred—and it is very doubtful if it was really on heat. Both in those sheep treated with stilboestrol alone, and in those given stilboestrol at or after gonadotrophin injection, the majority of those failing to ovulate came on heat, and the majority of those ovulating failed to come on heat. This is in contrast to the opinion of Quin & Van der Wath [1943] that oestrus following stilboestrol injection is an indication that ovulation has also been induced.

To obtain ovulation consistently stilboestrol alone is clearly inadequate; if serum gonadotrophin is used to obtain ovulation and stilboestrol given also, in order to induce heat, then, unless the stilboestrol dosage is below the pituitary threshold of sensitivity, it must be given after the gonadotrophin or it will inhibit ovulation. For this reason stilboestrol was given 24 hr. after serum gonadotrophin, and an attempt made to accelerate the onset of heat by giving large amounts and by various methods of administration. Even at this interval stilboestrol occasionally prevented ovulation.

Reference to Table 3 will show how these attempts failed. One animal, 163, came on heat within 12 hr. of intramuscular injection of 5 mg. of stilboestrol in suspension; it had in fact been served within  $4\frac{1}{2}$  hr. However, the remainder either failed to come on heat or came on after about the same interval as those given a subcutaneous injection in oil solution. Accordingly the remaining animals were given a small preliminary injection of stilboestrol di-*n*-butyrate, which is more slowly absorbed



than free stilboestrol. Most of these animals then received  $1\frac{1}{2}$  or 2 mg. of free stilboestrol a day after the gonadotrophin. The dose of dibutyrate proved to be not below the pituitary threshold, so corpora lutea were already present in some of these animals when the free stilboestrol was injected. In those which failed to ovulate this preliminary treatment seems not to have accelerated the onset of heat after the free stilboestrol was given.

Duration of heat was not measured; the intensity was not very great. There were two instances of unusual behaviour: 190 (Table 3) was licking the ram's face 36 hr. after stilboestrol injection, but refused to stand when he tried her, 201 (Table 2) was not served, but jumped other animals. Such 'male' behaviour seems to be due to prolonged stimulation rather than to heavy dosage.

### *Fertility*

Only two dividing ova were recovered. In cases where service occurred only a short time before killing it was not to be expected that division of the ovum would have started; however, most of the ova recovered were clearly degenerate, in a few cases only an empty zona being found. In most cases the timing of ovulation and heat was such that fertilization was very unlikely to occur.

The two dividing ova, both in the two-cell stage, were recovered from nos. 150 and 204. No. 150 was served between 2 and 3 days after being given 4 mg. of free stilboestrol; it was killed after 4 days. There were two ovulations, two ova were recovered from the tubes, the second being degenerate. No. 204 received 0.125 mg. of stilboestrol di-*n*-butyrate  $6\frac{1}{2}$  and 5 days before killing, gonadotrophin 4 days before killing, and 2 mg. of free stilboestrol a day later. 12-24 hr. before slaughter there was a doubtful service. In the left ovary there were two corpora lutea about 4 days old, in the right two corpora about 2 days old. In the right uterine horn there were two one-cell ova, not clearly degenerate in appearance; in the left tube there were two ova, one degenerate, the other an apparently normal two-cell ovum. Transperitoneal migration of ova seems not to offer a sufficient explanation; rapid ovum transport after the second lot of ovulations perhaps occurred. An ovum recovered from 162 was unevenly bilobed and irregular in shape; in 204 the cells were equal and regular.

It is noteworthy that none of 14 ova recovered after gonadotrophin injection and insemination were developing. This recalls the finding of Polovtsova & Judovitch [1939] that under these conditions the cervix is impassable to sperm.

### *Follicle growth*

Mention has already been made of large, tense, cystic-looking follicles in some animals treated with serum gonadotrophin and stilboestrol and which had not ovulated. Such follicles were seen in three other animals (one in each) which had ovulated. These were 199, treated with gonadotrophin alone, 190, with gonadotrophin followed by stilboestrol, and 197, given dibutyrate followed by gonadotrophin and later free stilboestrol.

Only one of those treated with stilboestrol alone had a large follicle when killed, and none of them a follicle of cystic appearance. The dose of serum gonadotrophin, 750 i.u., was probably well above the minimum effective level. Hammond *et al.* [1942] employed 400 i.u. on most animals and killed 4 or 5 days after injection. They

obtained ovulation with this amount in every case and found no obvious relationship between dose employed and extent of follicle stimulation; with a horse pituitary extract there was a clear gradation of response. In the cow [Hammond & Bhattacharya, 1944], with a similar interval between injection and killing, 100 mg. of this pituitary extract were found to be very roughly equivalent to 1500 i.u. of serum gonadotrophin in follicle-stimulating capacity.

This equation of relative potency does not agree with a comparison between the animals of the present series and those treated with horse pituitary by Hammond *et al.*, who obtained many large and medium-sized follicles after a single injection of 30–50 mg. of pituitary extract. It is noteworthy that these authors failed to reproduce with serum gonadotrophin treatment results obtained with horse pituitary extract injected during the normal breeding season, and that they considered this possibly attributable to inferiority of the animals used.

#### *Uterus and vagina*

The vaginal mucus was in nearly all cases of the thick cheesy type, irrespective of whether or not ovulation had been induced; there was no clear difference between groups differently treated. Uterine weights reveal little of interest; close comparison between groups is not possible because of the lack of uniformity among the animals used. Quin & Van der Wath [1943] comment upon the pale and inactive appearance of the uteri of some of their ewes after induced ovulation; many of the uteri in this series appeared similarly inactive.

#### DISCUSSION

For induced fertility in anoestrus to be practical commercially it is in general necessary for heat to be induced as well as ovulation, for in many circumstances artificial insemination will be inconvenient. A special problem arises in the sheep because of the absence of heat at the first normal, or gonadotrophin-induced, ovulation. Clearly it is desirable, if practicable, to obtain heat as well as ovulation with a single treatment because of the saving of work and time, and, if oestrogen is used as a supplement to the gonadotrophin, the elimination of a second treatment with the relatively more expensive gonadotrophin.

Potential fertility of ova shed after such treatment requires to be demonstrated; but, apart from such possible abnormality of ova, there would appear to be little prospect of obtaining such a treatment based upon serum gonadotrophin and stilboestrol. Reasons for this view concern the time relationship of oestrus and ovulation and are briefly as follows. If oestrogen is given later than serum gonadotrophin, it does not seem possible to obtain oestrus sufficiently early; whereas if given at the same time, or earlier, ovulation will either be induced before heat has time to develop, or else will be inhibited, unless the oestrogen dosage is below the pituitary threshold of sensitivity. The present results suggest that the level of dosage stimulating the pituitary is lower than that required to induce oestrus; absence of heat with gonadotrophin-induced ovulation would alone indicate this, were it not for the latent period between oestrogen administration and development of heat. Local administration of stilboestrol may perhaps be capable of inducing heat without also affecting the pituitary.

About the mechanism by which oestrous behaviour is hormonally provoked very little seems to be known. The fact that there is usually a latent period of about 2 days between administration of oestrogen and appearance of heat suggests that oestrogens act not directly upon the nervous system, but that a receptor requires first to be developed. The report of Polovtsova & Judovitch [1939] that tonic uterine contractions, present at oestrus, were absent at induced ovulation without heat suggests the particular possibility that proprioceptive elements in the uterus are involved.

McKenzie & Terrill [1937] treated spayed ewes with oestradiol benzoate, and Quin & Van der Wath [1943] treated with stilboestrol ewes spayed a year previously; in both cases oestrus was induced with greater consistency than in intact anoestrous ewes. McKenzie & Terrill found the histological state of the genital tract of the treated spayed animals 2 days after injection to approach that of normal oestrous ewes. The induction of ovulation in some of the intact animals, which in the present investigation was found usually to be unaccompanied by heat, is probably sufficient explanation for the greater sensitivity of castrate animals; while the very rapid recovery of the genital tract after oestrogen treatment shows that induction of heat in castrate ewes is not inconsistent with the possibility that oestrous behaviour is consequent upon uterine activity.

Evocation of heat in sheep by gonadotrophins is usual only if given in the presence of a regressing corpus luteum; previous sensitization of a receptor, or a direct oestrogen-progesterone synergism in induction of oestrous behaviour, are possible modes of action of the regressing corpus luteum. That either possibility is unlikely is indicated by the failure of Hammond *et al.* [1942] to obtain heat by previous treatment with progesterone, and by the lack of association of oestrus with ovulation when oestrogen is given. That the effect is due to the action of the corpus luteum in delaying ovulation is suggested by the latent periods between injection and ovulation, and between injection and manifestation of heat; the former appearing to be shorter in anoestrous sheep.

Lack of potential fertility in ova shed by anoestrous ewes after gonadotrophin treatment, and from ovulations induced in midcycle, is claimed by Murphree *et al.* [1944]. They used in most cases a series of daily injections of a follicle-stimulating sheep pituitary extract, followed by a luteinizing extract and insemination. As insemination was done 5 days after the first injection, and as large numbers of corpora lutea were found, it seems not unlikely that ovulation occurred some time before insemination, followed by a further series when the luteinizing extract was given. If so, it is not surprising if the first ova were unfertilized, while later ova would more properly be classified as 'luteal' (midcycle) than as 'anoestrous'. In the absence of data on sperm transport and times of ovulation, their findings cannot be considered conclusive. The failure to obtain fertilized ova after insemination in the present investigation can equally well be explained by failure of sperm transport in such cases, as reported by Polovtsova & Judovitch [1939].

Action of mare serum gonadotrophin in inducing ovulation is apparently dependent upon intervention of the pituitary of the treated sheep; this may also be true of follicle growth. It is suggested by the relatively slight extent of follicle growth obtained with serum gonadotrophin compared to horse pituitary extract when

given in dosage found roughly equivalent over a similar interval in the ewe. It might account for the failure of Hammond *et al.* [1942] to obtain a graded response to dosage, and is also suggested by experience in treatment of anoestrous cattle with serum gonadotrophin. 1500 i.u. of serum gonadotrophin is usually sufficient to obtain follicle growth and ovulation, but in some cases 5000 i.u. may give very slight follicle growth only, although [Hammond & Bhattacharya, 1944; Folley & Malpress, 1944] in oestrous cattle this amount will (with considerable individual variation) cause formation of very many large follicles. That the functions of follicle-stimulating and luteinizing hormones in bringing about follicle growth and ovulation may differ among different species is suggested by the absence of large follicles in the ovary of the pregnant mare, and by the finding of Rowlands & Williams [1943] that ovulation can frequently be induced by serum gonadotrophin in hypophysectomized rats.

## SUMMARY

Anoestrous sheep were treated with mare serum gonadotrophin and stilboestrol and slaughtered within a week of injection; ovaries were examined and search made for ova. All were run with a marked ram; those treated with serum gonadotrophin only were inseminated. Others were treated with stilboestrol only, or with stilboestrol by various methods of administration at intervals before and after serum gonadotrophin administration, and were not inseminated.

All animals treated with serum gonadotrophin alone had ovulated, none of them came on heat, and none of the ova recovered was fertilized. Ovulation was induced by stilboestrol alone only in a proportion of cases, probably those having already a fair-sized follicle present. Stilboestrol is capable of inhibiting the induction of ovulation by serum gonadotrophin if given before, or too soon after, administration of the gonadotrophin.

Heat was evoked by stilboestrol, alone or in combination with serum gonadotrophin, more frequently in those animals which did not ovulate. When both heat and ovulation occurred, their timing seemed such as to render fertilization improbable; only two fertilized ova were recovered. It appears that the interval between stilboestrol or serum gonadotrophin injection and ovulation is shorter than the latent period required in the development of heat.

There is some discussion on the bearing of these results upon prospects of commercial application to sheep breeding, upon the mechanisms involved in stimulation of ovulation and heat, and on the potential fertility of the ova obtained.

Thanks are due to those officials of the Ministry of Food who made available the animals used; to Dr J. H. Schulmann for suggestions upon methods of administering stilboestrol; to Dr M. C. Chang for performing inseminations; to Dr W. Garcia Vidal and Mr R. Mane Nin for other assistance. The mare serum gonadotrophin used was given by Messrs Boots, the British Drug Houses, and Organon, and the progesterone by the British Drug Houses. The work was carried out during tenure of a grant from the Agricultural Research Council.

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# INDUCTION OF LACTATION IN GOATS AND COWS WITH SYNTHETIC OESTROGENS AND ANTERIOR-PITUITARY EXTRACTS

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(Received 13 October 1944)

The stimulation of udder growth in virgin goats by inunction of the udder with ointment containing oestradiol monobenzoate, followed by lactation when the oestrogen treatment was replaced by injections of prolactin, was reported by de Fremery [1938]. Later, Folley, Scott Watson & Bottomley [1940, 1941] found that not only mammary development but also the initiation of copious lactation in virgin goats resulted from treatment with the synthetic oestrogen, diethylstilboestrol, alone. These results were confirmed in the goat by Lewis & Turner [1940, 1941] and in the bovine by Walker & Stanley [1940], Reece [1943], Folley & Malpress [1944*a*, *b*] and Hammond, Jr. & Day [1944].

De Fremery clearly states that in his experiments the oestrogen treatment merely promoted udder development, treatment with prolactin being necessary for the initiation of lactation. It is not yet clear whether or not diethylstilboestrol and its analogues differ from the natural oestrogens as Lewis & Turner [1940] affirm, in possessing the property of causing the initiation of lactation, presumably as a result of stimulation of the anterior pituitary.

In view of the marked galactopoietic activity of crude extracts of ox anterior pituitary [Folley & Young, 1938, 1939, 1940], it was of interest to study the initiation of lactation by combined treatment with synthetic oestrogens and anterior-pituitary extract, and a preliminary account of experiments along these lines [Folley & Young, 1941*a*] indicated that in some cases better results might be expected from the combined treatment than from the use of oestrogen alone. A more complete account of the above-mentioned experiments, together with a report of further experiments on the induction of lactation by combined treatment with synthetic oestrogens and anterior-pituitary extracts in goats, heifers and cows, is given in the present communication. Lewis & Turner [1942] have since reported experiments on the hormonal initiation of lactation in goats in which anterior-pituitary extracts were used in conjunction with diethylstilboestrol.

## MATERIALS AND METHODS

### *Animals*

The following animals were used.

(*a*) Two non-pedigree virgin female goats, G17 (Fanny) and G35 (Jemima), which had already undergone one hormonally induced lactation [see Folley *et al.* 1941]. These goats were approximately 3 and 2 years old respectively and were dry at the start of the present experiments.

(b) A group of six non-pedigree virgin goats originally comprising three pairs of twins; one animal, however, died before the experiment began and was replaced by another goat of almost the same age. Particulars of these goats are given in Table 1. These goats were dry at the time of the experiment and exhibited little udder development.

(c) Two maiden heifers of unknown breeding, one of Shorthorn type and the other resembling a Red Poll.

(d) Four cows, all of which were dry at the time experimental treatment began and which were made available on account of barrenness. Particulars of these heifers and cows are given in Table 2.

Table 1. *Details of treatment of virgin goats brought into lactation by implantation of oestrogen tablets or by oestrogen implantation combined with injections of ox anterior-pituitary extract*

Goat no.*	Age at implantation months	Wt. at implantation lb.	Tablets implanted mg.	Oestrogen	Implantation period days	Injections of anterior-pituitary extract beginning on the day of implantation
{ G 66	9	52½	8 × 50	Hoxoestrol	119	2.5 ml. daily for 183 days followed by 5.0 ml. daily for 72 days
{ G 67	9	58	—	—	—	
{ G 65	7	48½	8 × 50	Hexoestrol	119	—
{ G 64	7	46½	—	—	—	—
G 70	9	45½	8 × 50	Diethylstilboestrol	119	2.5 ml. daily for 183 days followed by 5.0 ml. daily for 69 days
G 69	9	45½	8 × 50	Diethylstilboestrol	119	

\* Twins are bracketed together.

Table 2. *Particulars of maiden heifers and dry cows treated with hexoestrol implants and injections of ox anterior-pituitary extract*

Animal no.	Heifer or cow	No. of calves	Hexoestrol tablets implanted mg.	Implantation period days	No. of injections of 10 ml. of anterior-pituitary extract on alternate days	Period between implantation and first injection days	Maximum daily milk yield
VL4	Heifer	0	20 × 50	102	20	102	12½ lb.
VL5	Heifer	0	1 × 1000	102	20	102	15 lb.
VL30	Cow	2	10 × 50*	70	11	29	1½ lb.
VL14	Cow	2	2 × 1000	55	12	40	50 ml.
VL16	Cow	2	2 × 1000	56	8	56	23 lb.
VL8	Cow	2	5 × 1000	63	17	71	50 ml.

\* These tablets consisted of 50 % hexoestrol and 50 % lactose.

### *Experimental treatments*

*Oestrogen.* For the inunction experiments an ointment containing 1 % diethylstilboestrol was prepared as described previously [Folley *et al.* 1941] and the requisite amount applied to the udder three times weekly.

Compressed tablets of pure *meso*-hexoestrol or diethylstilboestrol were used in the implantation experiments with the exception of one case in which tablets containing 50 % hexoestrol and 50 % lactose [cf. Folley, Stewart & Young, 1944] were used. They were subcutaneously implanted in the neck or flank as described by Folley *et al.* [1941] for the goat and Folley & Malpress [1944a] for the bovine.

In the experiments with goats there was a tendency for the tablets to become extruded soon after implantation. In each case, as soon as the trouble was noticed, such tablets as were still *in situ* were removed and new tablets implanted into a fresh site. This, of course, necessitated the removal at the same time of the tablets from the other member of the pair, i.e. the goat receiving the same oestrogen treatment, and the implantation of new tablets. In one experiment dienestrol was orally administered, incorporated in cattle cubes as described previously [Folley & Malpress, 1944b].

*Anterior-pituitary extract.* A crude alkaline extract of ox anterior pituitary [Young, 1941] was injected subcutaneously. Ten ml. of this extract were equivalent to 2.5 g. of fresh anterior-lobe tissue.

#### *Milk analyses*

Analytical methods were as used previously [Folley & Malpress, 1944c].

### RESULTS

#### *Milk yield*

##### *Inunction experiments*

The two goats, G 17 and G 35, were used in these experiments and at the outset given treatment consisting of three inunctions weekly with 1 g. of 1 % diethylstilboestrol ointment. The lactation curves are given in Fig. 1.

In the case of G 17 the oestrogen inunctions caused, in contradistinction to the first time she was artificially brought into milk [Folley *et al.* 1941], the production of traces of secretion only; doubling the dose for a short interval caused no further response. The original dose was restored, still without result, but the institution of injections of 5 ml. of anterior-pituitary extract on alternate days at once brought the goat into lactation and her yield quickly rose to a peak of almost 1700 ml. daily, a value somewhat greater than that attained during the previous lactation. The yield declined rapidly from the peak value and the decline was not arrested by stopping the oestrogen treatment, even though the anterior-pituitary injections were continued. The cessation of anterior-pituitary treatment was followed by a precipitous fall in milk yield to a level of about 900 ml. daily, but thereafter, during the autumn and winter months, the decline was much more gradual. A marked rise in yield occurred without further treatment in the following spring. When the yield had attained its new maximum, daily injections of 5 ml. of anterior-pituitary extract were begun; sixty-six injections were given in all, spread over a period of 76 days. This treatment at first caused a further rise in milk production, but soon the yield began to decline rapidly. The goat was now seen to be ill and she died 76 days after the start of the second course of injections.

The first time she was used in an experiment on the hormonal induction of lactation [Folley *et al.* 1941], G 35, then 8 months old, required some 4 months' inunction (with diethylstilboestrol dipropionate) before she came into lactation and then her milk yield increased quite slowly to a maximum of 600 ml. daily. By contrast, she now came into lactation soon after inunction began and her yield rose rapidly to a peak value of over 500 ml. daily. Injections of 5 ml. of anterior-pituitary extract on alternate days, begun just after the peak of lactation had been passed, resulted



in a further increase to a new maximum of almost 900 ml. daily. As in the case of G17, the yield declined rather rapidly from this peak, but the decline was less rapid after stopping oestrogen treatment. When anterior-pituitary injections ceased, the yield fell rapidly to a level roughly corresponding to that expected had it declined at a normal rate from the first maximum. Subsequently an attempt was made to increase the yield by further oestrogen treatment [see Walker & Stanley, 1941], a single 1000 mg. tablet of diethylstilboestrol being implanted subcutaneously. The initial absorption from this tablet, however, was possibly great enough to exert

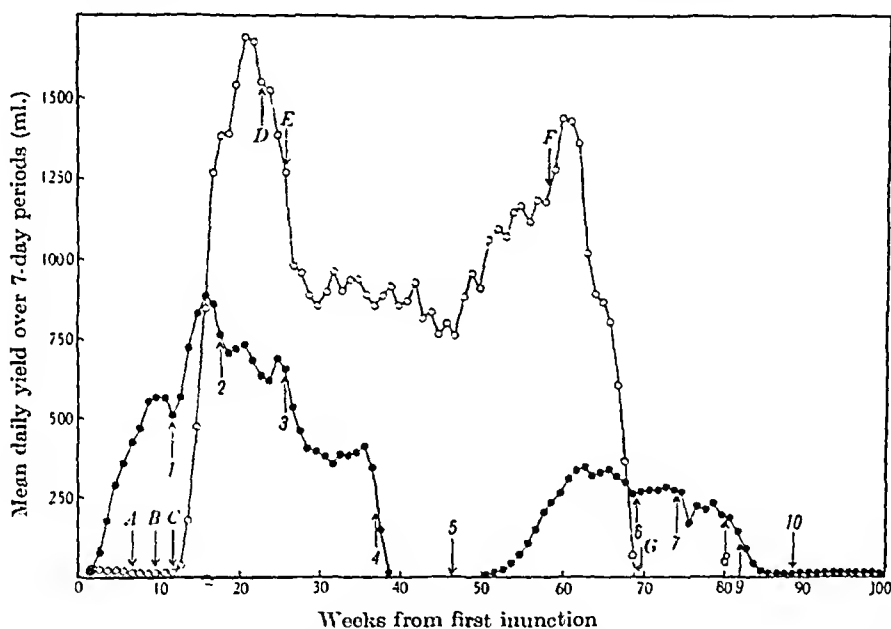


FIG. 1. Lactation curves of virgin goats brought into lactation with diethylstilboestrol (by udder inunction) and anterior-pituitary extract. O—O, goat G17. Origin: inunction three times weekly with 1 g. of 1% ointment begun. A, dose of oestrogen increased to 2 g. B, original dose restored. C, injections of 5 ml. anterior-pituitary extract (1.25 g.-equiv.) on alternate days begun. D, inunctions ceased. E, injections ceased. F, daily injections of 5 ml. of anterior-pituitary extract begun. G, injections ceased; goat died on the day following the last injection. ●—●, goat G35. Origin: inunction three times weekly with 1 g. of 1% ointment begun. 1, injections of 5 ml. anterior-pituitary extract (1.25 g.-equiv.) on alternate days begun. 2, inunctions ceased. 3, injections ceased. 4, subcutaneous implantation of  $1 \times 1000$  mg. tablet of diethylstilboestrol. 5, tablet removed. 6, oral administration of 20 mg. of dienioestrol on alternate days begun. 7, dose of dienioestrol increased to 20 mg. daily. 8, dose of dienioestrol increased to 40 g. daily. 9, dose of dienioestrol increased to 80 mg. daily. 10, end of feeding period.

an inhibitory rather than a stimulating effect, for the goat went dry soon after implantation. About 2 weeks after the removal of the tablet she came slowly into milk once more, the slow rise in yield probably being correlated with the gradual elimination from the body of accumulated oestrogen. Once more the level reached was that which might have been expected had no treatment been instituted after the initial inunction. An attempt was later made to cause further stimulation of lactation by the oral administration of dienioestrol. The milk yield was slowly declining when feeding began, and the curve suggests that 20 mg. every other day caused an interruption of the decline. Progressive increases in the dosage, however, quickly caused inhibition and the goat became, to all intents and purposes, dry.

*Implantation experiments*

*Goats.* Particulars of the six goats used in these experiments and details of their experimental treatment are given in Table 1. In view of the tendency for pedigree virgin females of high milking strains to lactate spontaneously, it was considered advisable, even though in our experience this rarely happens with 'scrub' females, that one member of each of the two pairs of twins should serve as an untreated control to its treated twin. Attempts to obtain milk from the controls were begun simultaneously with the milking of the experimental animals, but neither gave any milk during the experiment. Owing to the partial extrusion of the oestrogen implants one re-implantation with new tablets was necessary in G66 and two in G69 before

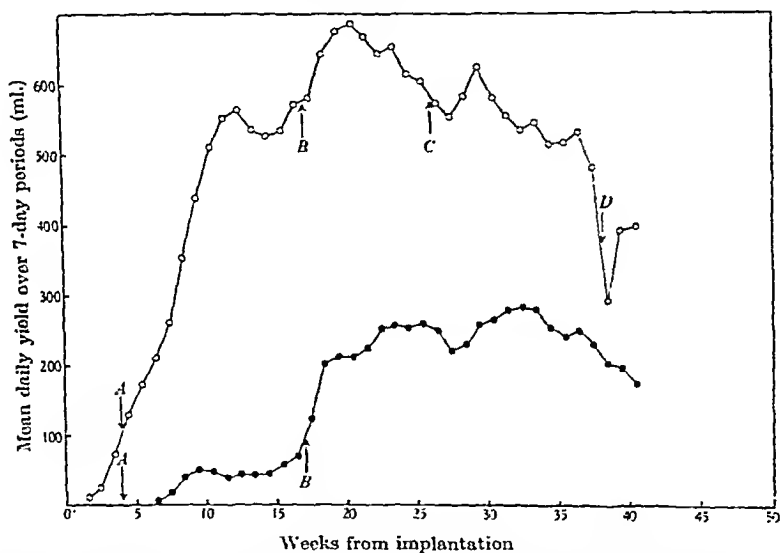


Fig. 2. Lactation curves of virgin goats brought into lactation by subcutaneous implantation of hexoestrol tablets alone, or combined with injections of anterior-pituitary extract. O—O, goat G66, subcutaneously implanted with  $8 \times 50$  mg. hexoestrol tablets at which time daily injections of 2.5 ml. of anterior-pituitary extract (0.625 g.-equiv.) were begun. ●—●, goat G65, subcutaneously implanted with  $8 \times 50$  mg. hexoestrol tablets. Origin: tablets implanted and injections (G 66) begun. A, new tablets implanted into G65 since original implant was extruded; at the same time the implant was removed from G 66 and replaced with new tablets. B, tablets removed. C, daily dose of anterior-pituitary extract doubled. D, injections ceased.

the date fixed for the final removal of the implants. This necessitated the removal of the implants from G65 and G70 on the appropriate occasions and the re-implantation of new tablets (see Figs. 2, 3). Injections of anterior-pituitary extract began on the day of implantation.

The lactation curves of G65 and G66 (hexoestrol implants) are given in Fig. 2 and of G69 and G70 (diethylstilboestrol implants) in Fig. 3. It will be seen that in both cases the goat receiving anterior-pituitary extract came into milk before the corresponding animal treated with oestrogen only. Further, it may be noted that the anterior-pituitary-treated goat in each instance gave larger maximum and total yields. The curve of G70 is of interest in that on the first occasion on which she

was re-implanted her yield, which was rising rapidly at the time, was immediately depressed. The corresponding re-implantation of G69 had no such effect, nor did the re-implantation of G66 (see Fig. 2). It seems likely that in some way the first re-implantation of G70 resulted in a sudden and perhaps transient increase in oestrogen dosage sufficient to inhibit lactation. The superiority of combined treatment

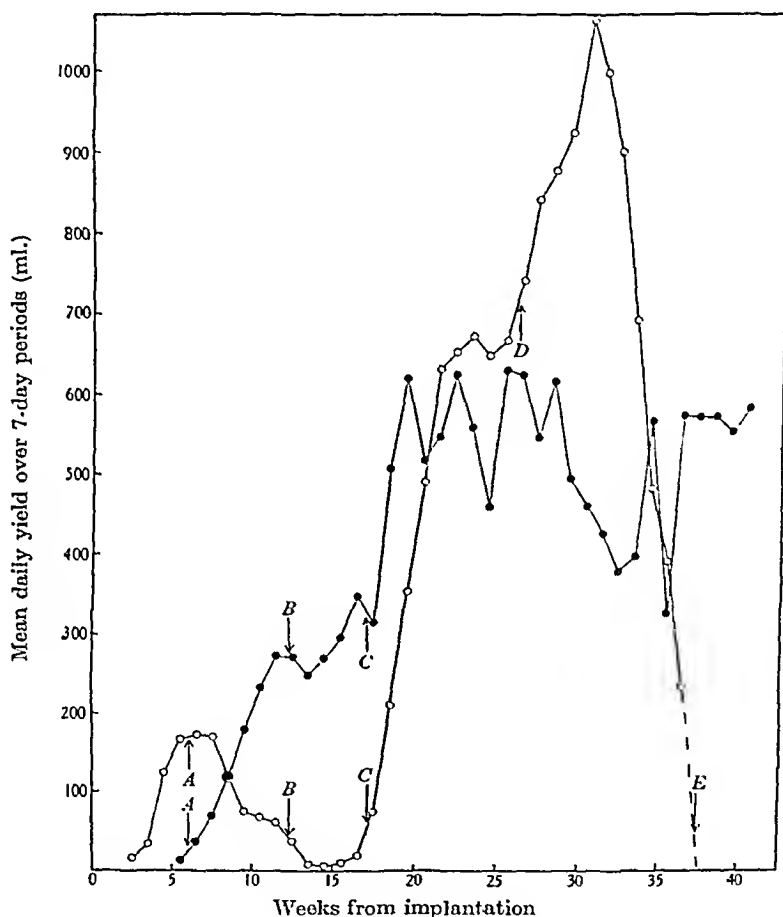


FIG. 3. Lactation curves of virgin goats brought into lactation by subcutaneous implantation of diethylstilboestrol tablets alone, or combined with injections of anterior-pituitary extract.  $\bigcirc$ — $\bigcirc$ , goat G70, subcutaneously implanted with  $8 \times 50$  mg. diethylstilboestrol tablets at which time daily injections of 2.5 ml. of anterior-pituitary extract (0.625 g.-equiv.) were begun.  $\bullet$ — $\bullet$ , goat G69, subcutaneously implanted with  $8 \times 50$  mg. diethylstilboestrol tablets. Origin: tablets implanted and injections (G70) begun. *A*, new tablets implanted into G69 since original implant was extruded; at the same time the implant was removed from G70 and replaced with new tablets. *B*, new tablets implanted into G69 since the second implant was extruded; at the same time the implant was removed from G70 and replaced with new tablets. *C*, tablets removed. *D*, daily dose of anterior-pituitary extract doubled. *E*, injections ceased; goat died 2 days after the last injection.

with oestrogen and anterior-pituitary extract over treatment with oestrogen alone was most marked in the experiment on G65 and G66. It should be noted that G66, the goat receiving anterior-pituitary extract, was 2 months older than G65, which might account for part of the difference in response; on the other hand, at the time of the experiment, the goats differed in weight by only 4 lb. (Table 1).

It may be concluded that the oestrogen dosage in all four cases was above optimal, since removal of the implant was followed in each case by an immediate and unmistakable increase in milk yield. Increasing the daily dose of anterior-pituitary extract to 5 ml. had little effect on the milk yield of G66, but in G70 caused a further marked increase (Fig. 3). After some weeks of treatment at the higher level, however, both goats became ill and their milk yields decreased, catastrophically in the case of G70. After stopping the injections G66 recovered but G70 died.

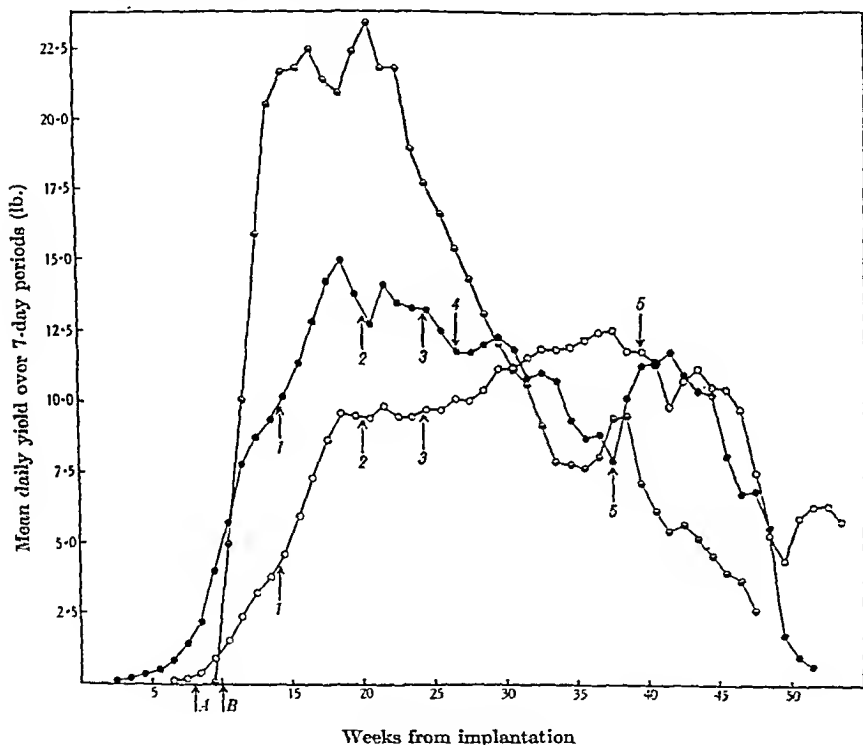


FIG. 4. Lactation curves of two nulliparous heifers and one dry cow which received subcutaneous implants of hexoestrol tablets and injections of anterior-pituitary extract. O—O, heifer VL4, subcutaneously implanted with 20 × 50 mg. hexoestrol tablets. ●—●, heifer VL5, subcutaneously implanted with 1 × 1000 mg. hexoestrol tablet. Origin: tablets implanted. 1, tablets removed and daily injections of 10 ml. of anterior-pituitary extract (2.5 g.-equiv.) on alternate days begun. 2, injections ceased. 3, 1 × 1000 mg. hexoestrol tablet subcutaneously implanted. 4, tablet removed and a similar tablet implanted into a fresh site (VL5 only). 5, tablets removed. ○—○, cow VL16, subcutaneously implanted with 2 × 1000 mg. hexoestrol tablets. Origin: tablets implanted. A, tablets removed and injections of 10 ml. of anterior-pituitary extract (2.5 g.-equiv.) on alternate days begun. B, injections ceased.

Some 9½ months after the beginning of the main experiment, a similar experiment was carried out on the two untreated controls, G64 and G67, each goat receiving the same initial treatment as her twin had earlier received, even to the renewal of the hexoestrol implants at the appropriate time after the original implantation. Once again the anterior-pituitary-treated goat (G67) came first into lactation; the first traces of secretion were obtained from her by the second week after implantation

and her yield rose steadily to a value of about 260 ml. daily at the 5th week. At this point the yield began to decrease sharply and it was noticed that the animal was ill. The injections were accordingly discontinued 44 days after they began, despite which the goat died 11 days later. The goat receiving oestrogen only (G 64) failed to respond to treatment; she gave no mammary secretion until the 6th week after implantation and thereafter never more than 20 ml. The failure of this goat to respond at the age of 16½ months to a treatment identical with that which induced lactation in her twin at the age of 7 months is difficult to explain. It is possible that the threshold for the oestrogenic stimulus to lactation changes as the animal matures, so that a dose which is effective in a young animal may not be suitable for an older one.

*Heifers and cows.* Table 2 gives details of the animals and their treatment. The lactation curves of the three animals which gave appreciable yields of milk are shown in Fig. 4. In the two heifers, VL4 and VL5, lactation was initiated following the implantation of hexoestrol tablets, and the tablets were removed and injections of anterior-pituitary extract begun as soon as the lactation curves began to flatten out. As a result, the lactation curves immediately rose sharply once more; it seems clear that in both cases the injections of anterior-pituitary extract increased the peak yield over the value expected from treatment with oestrogen alone, though it must be admitted that in either case the increase in yield might have been due primarily to removal of the tablets. Cessation of the injections after the yields had become steady was not followed by any marked changes in milk secretion. Later an attempt was made further to boost the yields of both heifers by oestrogen treatment. Each received an implant of a single 1000 mg. tablet of hexoestrol. In the case of VL5 the implantation site became inflamed shortly after the operation and the tablet was removed and a new one re-implanted into another site. In VL4 the implantation was followed by a steady increase in yield for a considerable period, while in VL5 the treatment had the opposite effect. Conversely, the two heifers reacted differently to the removal of the tablets, the yield of VL4 showing a fall while that of VL5 rose sharply.

The dry barren cow, VL16, gave no milk whatever during the oestrogen implantation period of 8 weeks. She was given a short series of injections of anterior-pituitary extract beginning on the day the implant was removed, and she quickly came into milk in response to this stimulus. Her yield increased rapidly to a peak of 23 lb. daily but then fell off rather sharply.

#### *Milk composition*

Analyses were carried out at regular intervals on the secretions given by the goats and the two heifers. The present results are similar to those reported previously in experiments on the artificial induction of lactation in goats [Folley *et al.* 1941] and bovines [Folley & Malpress, 1944c]. Typical results for milk samples taken from the goats only are given in Table 3. They indicate that a yield of 500 ml. daily, and often much less, is a guarantee of normal composition.

Table 3. *Composition of mammary secretions from virgin goats brought into lactation by combined treatment with oestrogen and anterior-pituitary extract or by treatment with oestrogen only*

No.	Days after first treatment	Daily milk yield ml.	Fat %	S.N.F. %	Total N mg. %	Casein N mg. %	Non-protein N mg. %	Casein no.*	Lactose %	Cl mg. %
†	—	—	4.50	9.02	522	387	—	74.2	4.08	105
G66	98	562	4.43	9.16	579	473	37	81.7	4.36	95
G65	196	222	3.70	9.55	644	469	42	75.9	—	—
G70	161	690	4.20	9.08	572	388	57	67.8	—	—
G69	161	596	4.20	10.56	748	554	50	74.1	—	—
G17	104	548	3.83	9.67	653	483	34	73.9	4.62	145
G35	104	810	4.70	9.33	545	385	30	70.7	4.74	115

\* Casein no. =  $\frac{\text{casein N}}{\text{total N}} \times 100$ .

† Average composition of goats' milk under English conditions [Knowles & Watkin, 1938].

### Absorption data

The absorption data relating to the implants into goats are given in Table 4 and those relating to the implants into heifers and cows in Table 5. No determinations

Table 4. *Absorption data for goats implanted with synthetic oestrogens*

Goat no.	Implant*	Figure reference to implant	Wt. of implant mg.	Implan-tation period days	Wt. of recovered implant mg.	Total absorption mg.	Mean daily absorption mg.
G66	8 × 50 mg. Hex.	A, Fig. 2	394.2	91	331.9	62.3	0.7
G65	8 × 50 mg. Hex.	A, Fig. 2	401.0	91	255.3†	109.0†	1.2†
G64	8 × 50 mg. Hex.	—	362.0	98	193.0	169.0	1.7
G70	8 × 50 mg. Daes.	A, Fig. 3	396.3	42	354.9	41.4	1.0
G70	8 × 50 mg. Daes.	B, Fig. 3	398.6	33	370.5	28.1	0.9
G69	8 × 50 mg. Daes.	B, Fig. 3	392.6	33	355.0	37.6	1.1
G35	1 × 1000 mg. Daes.	4, Fig. 1	896.0	64	815.6	80.4	1.3

\* Hex. = hexoestrol; Daes. = diethylstilboestrol.

† Only seven tablets recovered.

‡ Estimated absorption.

Table 5. *Absorption data for heifers and cows implanted with hexoestrol*

Animal no.	Hexoestrol tablets implanted mg.	Figure reference to implant	Wt. of implant mg.	Implan-tation period days	Wt. of recovered implant mg.	Total absorption mg.	Mean daily absorption mg.
VL4	20 × 50	Origin, Fig. 4	1104.0	102	742.5	361.5	3.5
VL4	1 × 1000	3, Fig. 4	1011.3	105	734.9	276.4	2.6
VL5	1 × 1000	Origin, Fig. 4	1121.0	102	849.1	271.9	2.7
VL5	1 × 1000	3, Fig. 4	1043.8	16	914.8	129.0	8.1
VL5	1 × 1000	4, Fig. 4	962.6	77	758.2	204.4	2.7
VL30	10 × 50*		477.9	70	137.7	101.3†	1.4
VL14	2 × 1000		1920.0	55	1675.0	245.0	4.5
VL16	2 × 1000	Origin, Fig. 4	1910.0	56	1620.0	290.0	5.2
VL8	5 × 1000		5240.0	63	4380.0	860.0	13.7

\* 50% hexoestrol and 50% lactose.

† Excluding lactose absorption.

of the weights of the ghosts [Folley, 1944] were made, so that the absorption rates, particularly in the case of 1000 mg. tablets, are likely to be somewhat lower than the true values. The results in Table 4 do not include data for every implantation into goats because at first the tablets were not accurately weighed before implantation. Moreover, in three cases insufficient tablets were recovered to make possible the calculation of valid absorption values. Some of the results given in Table 5 have already been reported in another connexion [Folley & Malpress, 1944a].

The only feature of these results calling for comment is the unusually high absorption rate of the second implant in VL5; this is undoubtedly due to the initial absorption of the labile component [Folley, 1944], the effect of which on the mean absorption rate is particularly important over short implantation periods.

*Possible pathological effects of prolonged treatment  
with anterior-pituitary extract*

Three goats, G 17, G 66 and G 70, which had received prolonged treatment with anterior-pituitary extract, eventually and simultaneously showed symptoms of acute illness. Two of them soon died, but the third (G 66), which was treated with lamb dysentery serum, recovered. About 3 months later a fourth goat (G 67) had a similar fatal illness after a relatively short course of injections of anterior-pituitary extract. In no case were the post-mortem findings very definite, but they were not inconsistent with a diagnosis of enterotoxaemia caused by infection with organisms of the *welchii* group. The intestinal and stomach contents were, however, not examined for the presence of *Cl. welchii* toxins. It is noteworthy that among the experimental herd only those goats receiving anterior-pituitary extract showed at this time any untoward symptoms, so that it seems probable that the condition was associated with the anterior-pituitary treatment. It is impossible to say, however, whether the condition was due to a direct action of the anterior-pituitary extract, which was sterile, or whether the extract increased the susceptibility of the goats to *Cl. welchii* infection.

DISCUSSION

The present results fall into three categories. First, there are two cases in which lactation was quickly initiated by treatment with anterior-pituitary extract after prolonged oestrogen treatment had evoked no lactational response. Secondly, there are three experiments in which lactation was more quickly initiated by combined treatment with oestrogen and anterior-pituitary extract than by oestrogen alone. Finally, in three experiments anterior-pituitary extract exerted a substantial, if temporary, galactopoietic effect at the peak of a lactation originally induced by oestrogen treatment.

These various types of result are explicable in the light of the different biological activities exhibited by the extracts used. Such extracts have been shown to be characterized by possession of three kinds of biological activity relevant to mammary gland physiology, namely, (a) mammogenic activity [Cowie & Folley, 1944], (b) lactogenic activity [Folley & Young, 1938] depending essentially on the presence of prolactin, but probably also needing the co-operative activity of other pituitary factors either present in the preparation tested or supplied by the test animal's own pituitary

[see Folley & Young, 1941*b*], and (c) galactopoietic activity [Folley & Young, 1938, 1939, 1940].

The hormonal induction of lactation by oestrogen may be explained as involving in the first place mammary growth, probably due to the synergistic action of oestrogen and anterior-pituitary mammogenic hormones produced in response to the oestrogenic stimulus. The latter also probably evokes the production by the anterior lobe of lactogenic and galactopoietic hormones responsible for the initiation and maintenance of lactation. It is probable that anterior-pituitary hormone complexes are involved in both these latter processes [see Folley & Young, 1941*b*], and while it is not possible at present to say whether or not they are identical, it is almost certain that some components are common to both.

In cases of artificial induction of lactation it is reasonable to suppose that the limiting factor governing the rate of increase in milk yield is the rate of growth of mammary tissue. The greater the latter, the sooner will lactation be initiated. The earlier induction of lactation in goats receiving simultaneous treatment with anterior-pituitary extract and oestrogen as compared with goats receiving oestrogen alone may therefore be ascribed to more rapid mammary growth due to the mammogenic properties of the anterior-pituitary extract acting in concert with the oestrogen [cf. Gardner & White, 1942].

In the cases in which treatment with anterior-pituitary extract initiated lactation after oestrogen treatment had failed to do so, it seems clear that though the oestrogen treatment had caused mammary development it had failed to influence sufficiently the release of lactogenic hormones by the anterior lobe. Though the onset of lactation in VL 16 might have been primarily due to the removal of the oestrogen tablets, it seems much more likely that here as in G 17 the necessary stimulus for the initiation of lactation was supplied by the exogenous extract. In previous experiments on bovines [Folley & Malpress, 1944*a, b*; Hammond, Jr. & Day, 1944] cases were encountered in which there was a complete failure to respond to oestrogen treatment, and it seems possible that these failures might be explained on the basis just outlined. The three negative results out of the four given in Table 2, however, indicate that by no means all animals which failed to respond to oestrogen would have responded had anterior-pituitary extract been administered.

The stimulation of lactation which resulted when anterior-pituitary extract was administered at the point of maximum yield resulting from oestrogen treatment can undoubtedly be ascribed to the action of the galactopoietic complex present in the anterior-pituitary extract. Since there is evidence from experiments on cows [Folley & Young, 1945] that this extract does not exert its galactopoietic action at the peak of lactation, it would seem that in the present experiments the peak yields resulting from treatment with oestrogen alone were not the maximum possible yields.

The present experiments provide further evidence in support of the concept [Folley, 1941] that as regards the secretion of hormones concerned in lactation, oestrogen in relatively low doses exerts a stimulatory effect on the anterior pituitary while the action of relatively high doses is inhibitory. A good example is the arrest in the decline in the milk yield of G 35 (Fig. 1) following the oral administration of 20 mg. of dienoestrol on alternate days and the rapid fall in yield when the dose was increased. Conversely, the same goat earlier had rapidly dried off following the



subcutaneous implantation of a single 1000 mg. tablet of diethylstilboestrol and began to secrete milk once more after the tablet had been removed. The experiments on G69 (Fig. 3) and G65 (Fig. 2) provide examples of an increase in milk yield following cessation of oestrogen treatment such as was observed in experiments on cows and heifers by Folley & Malpress [1944*a, b*]. The results following the implantation during declining lactation of the two heifers VL4 and VL5 with a single 1000 mg. hexoestrol tablet (Fig. 4) are also in harmony with this concept.

The results described in the present communication throw some light on the factors involved in the artificial induction of lactation. In particular, they indicate that for maximum response a delicate adjustment of the level of oestrogen in the circulation in relation to the activity of the anterior pituitary must be maintained. They do not, however, provide a complete explanation of the individual variability in response to oestrogen which has hitherto been so prominent a feature of experiments on bovines. Many problems connected with the hormonal induction of lactation remain unsolved, and further elucidation of the endocrine relationships involved must await further experimental work.

#### SUMMARY

1. Simultaneous treatment with oestrogen and ox anterior-pituitary extract induced lactation in virgin goats sooner than treatment with oestrogen alone.
2. In two out of five experiments on cows or goats in which prolonged oestrogen treatment failed to induce lactation, the institution of anterior-pituitary injections was followed by copious milk secretion.
3. Anterior-pituitary extract exerted a galactopoietic effect at the peak of lactation induced by oestrogen treatment in maiden heifers or virgin goats.

For co-operation in providing access to some of the cows used in this work we are indebted to two private owners, Messrs H. M. Budgett and W. Mackay, and to the late Major G. W. Dunkin, then Director of the Agricultural Research Council's Field Station. We also acknowledge gratefully the help of Mr R. E. Glover, F.R.C.V.S., who implanted oestrogen tablets into two of the animals, and Mr A. T. Cowie, M.R.C.V.S., for performing post-mortem examinations. Our thanks are also due to the Agricultural Research Council for a research grant to one of us (F. H. M.), and to the Medical Research Council for a special grant towards the expenses of this work.

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# THE GALACTOPOIETIC ACTION OF PITUITARY EXTRACTS IN LACTATING COWS

## 1. DOSE-RESPONSE RELATIONS AND TOTAL YIELDS DURING DECLINING LACTATION

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(Received 13 October 1944)

Our previous investigations of the influence of injected anterior-pituitary extract on the milk yield of cows in declining lactation have shown that over a period of 3 weeks such treatment may induce a 16–25% rise in milk production [Folley & Young, 1938, 1939, 1940]. The series of investigations now described was undertaken at the beginning of the present war to determine more precisely the conditions under which an increase of milk yield under pituitary stimulation could be consistently obtained, and to ascertain whether such a process provided a means of producing a significant increase in the total milk yield in Great Britain.

Before field trials were undertaken it was necessary to determine whether animals of known reactivity under first class management would consistently respond to treatment, to ascertain the minimum dose of pituitary extract required to induce an adequate response, and to estimate the minimum frequency of pituitary injection necessary to produce a sustained response. Opportunity was also taken in these exploratory experiments to determine whether whole pituitary glands and desiccated anterior-pituitary tissue would yield extracts of satisfactory potency for field trials.

### METHODS

#### *Animals*

Eighty-five cows in declining lactation were used in these preliminary experiments. Forty-eight of these, British Friesian and Ayrshire animals, belonged to the Agricultural Research Council's Field Station, Compton, Berks; twenty-nine animals of the South Devon breed were owned by Dartington Hall Ltd., Totnes, South Devon, and eight cows, of Dairy Shorthorn stock, were part of the herd of Reading University Farm, Sonning, Berks. Preliminary experiments had shown that animals of these several breeds were all responsive to the galactopoietic action of our anterior-pituitary extract. In most experiments they were treated in groups which, as in our previous investigations of this type, consisted of animals which were as similar as possible with respect to stage of lactation, time since mating, etc. They were milked twice daily, the milk being weighed to  $\frac{1}{4}$  lb.

#### *Anterior-pituitary extracts*

The standard concentration of our aqueous alkaline extract was such that 10 ml. contained the material extracted from 2.5 g. of fresh ox anterior-pituitary tissue; 10 ml. was the dose commonly administered, and it is described for convenience as

2.5 g.-equiv. of pituitary extract. Where an amount less than 2.5 g.-equiv. was administered the appropriate amount of standard extract was diluted with saline to 10 ml. for administration. After preparation the extracts were preserved in the refrigerator.

In most experiments control animals received treatment with an extract of calf thymus gland prepared in the same manner as the pituitary extract. The full results of the majority of these control experiments are not included because they merely indicated that the decline of milk production continued uninterruptedly throughout the experimental period at about the same rate as had been determined for the pre-experimental control period.

(a) *Aqueous alkaline extract of fresh anterior-lobe tissue.* This was similar to that used in our previous experiments [Folley & Young, 1940] and consisted of a crude extract of absolutely fresh ox anterior-pituitary tissue prepared at 0° by extraction with 0.9% NaCl solution at pH 8.5.

(b) *Aqueous alkaline extract of whole ox-pituitary tissue.* This was prepared in the same way, the final concentration being such that 10 ml. was equivalent to 2.5 g. of fresh anterior-lobe tissue.

(c) *Aqueous alkaline extract of desiccated anterior-lobe tissue.* Three different methods of desiccating fresh ox anterior-lobe tissue were used. In the first the frozen tissue was minced into 40 vol. of cold absolute acetone, and after the mixture had stood overnight at 0°, the precipitate was filtered off, washed with fresh acetone, and dried *in vacuo* over calcium chloride at room temperature. For the second method a similar procedure was followed with absolute ethanol instead of acetone, while for the third modification the desiccating fluid was commercial methylated spirit (Methcol Co. Ltd.).

In every case the dried tissue was extracted within a few days of desiccation, with two portions of alkaline (pH 8.5) 0.9% NaCl solution of such volume that 10 ml. of the final combined extracts contained the material extracted from 0.5 g. of dried material, which amount was considered to be equivalent to 2.5 g. of fresh tissue.

#### *Injection of extracts*

Extracts were always administered subcutaneously usually before or during the afternoon milking period.

### RESULTS

#### *Determination of the minimum effective dose of the crude extract of fresh anterior-lobe tissue*

*Results of a single injection of extract.* In our previous investigations we confirmed the finding of Asimov & Krouze [1937] that a single injection of a crude alkaline extract of fresh anterior lobe resulted in a substantial but temporary increase in the milk yield of cows in declining lactation, and we found that a measure of the galactopoietic activity of such extracts could be obtained by determining the difference between the average yields of groups of cows for 2 days before and 2 days after the injection of a single dose of pituitary extract, the increase being expressed as a percentage of the average pre-injection yield [cf. Folley & Young, 1940]. There is no reason to believe that the neutral saline pituitary extracts we used in our first experiments with cows [Folley & Young, 1938] differ in any way in their physiological effects from the alkaline saline extract we used subsequently [Folley & Young, 1939, 1940]; the combined results of our earlier investigations therefore suggest that 5 g.-equiv. (neutral extract) and 2.5 g.-equiv. (alkaline extract) both give an effect which is almost maximal [Folley & Young, 1940].

We have therefore determined the influence of treatment with a single dose of extract equivalent to 2.5, 1.25 or 0.625 g., of anterior lobe, on large groups of cows in declining lactation, in each case comparing the result with that from similar animals treated with thymus extract. The results (Table 1) show that 1.25 g.-equiv.

Table 1. *Influence of a single subcutaneous injection of an extract of ox-pituitary tissue on the milk yield of cows in declining lactation*

Tissue extract used	Amount administered (g.-equiv. of fresh tissue)	No. of cows in group	Milk yield			Not increase due to pituitary extract %
			Mean for 2 days before injection lb./cow/day	Mean for 2 days after injection lb./cow/day	Increase %	
Anterior pituitary	2.5	16	23.1	25.4	10.0	7.7
Thymus	2.5	16	21.7	22.2	2.3	
Anterior pituitary	1.25	10	20.7	21.6	4.3	4.3
Thymus	2.5	5	21.2	21.2	0.0	
Anterior pituitary	0.625	10	21.8	21.0	0.5	0.5
Stale* anterior pituitary	2.5	4	16.8	18.3	8.9	12.9
Nil	—	3	17.7	17.0	-4.0	
Whole pituitary	2.5	4	9.5	10.5	10.5	—
Anterior pituitary	2.5	4	8.0	8.7	8.7	

In every case the volume of extract administered was 10 ml./cow.

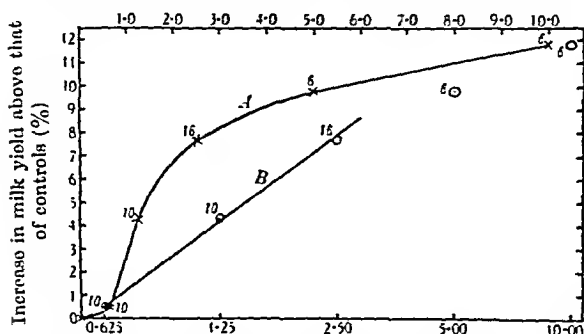
\* Crude suspension kept at room temperature for 24 hr.

gave a much smaller effect than did 2.5 g.-equiv., while 0.625 g.-equiv. had no significant effect. In a previous investigation we found [Folley & Young, 1938] that a single injection of 5.0 g.-equiv. of anterior-pituitary extract increased the mean milk yield for a 2-day period by 9.8%, while with 10.0 g.-equiv. the increase was 11.8%. Thus the 7.7% increase found with 2.5 g.-equiv. in the present experiments was not maximal but was obviously substantial for the relatively small dose administered.

In Fig. 1 the data from the present experiments, together with those from the experiments of Folley & Young [1938] referred to above, are plotted; curve *A* shows the relation between the increase in milk yield and the dose of extract administered, while curve *B* shows that between the increase in yield and the logarithm of the dose of pituitary extract injected. These results suggested that a dose of 2.5 g.-equiv. of fresh tissue was worth further investigation in that it stood towards the top of the steep part of the sigmoid curve *A* (arithmetic) and was the highest point on the straight-line portion of the logarithmic curve *B*. Experiments were accordingly undertaken to determine the influence of repeated injections of this dose of extract.

*Results of repeated injections of a dose of extract equivalent to 2.5 g. of fresh anterior-lobe tissue.* We have previously shown that the repeated administration of pituitary extract at 2-day intervals results in a substantial and prolonged enhancement of milk production in cows in declining lactation [Folley & Young, 1939, 1940]. We accordingly first tested different doses of extract injected at 2-day intervals, giving eleven injections over a period of 21 days. The results (Fig. 2) showed that a substantial increase in milk production was obtained with repeated doses of 2.5 and 1.25 g.-equiv., under these conditions, but not with 0.625 g.-equiv.

Extract administered (g.-equivalent of fresh pituitary tissue)



Extract administered (logarithmic scale) (g.-equivalent of fresh pituitary tissue)

FIG. 1. The influence of a single injection of anterior-pituitary extract on the milk yield of groups of cows in declining lactation. The figures attached to the points give the number of cows in the group. Curve A, mean daily increase in milk yield over 2 days resulting from treatment, plotted against the dose of extract given. Curve B, mean daily increase in milk yield over 2 days resulting from treatment, plotted against the logarithm of the dose of extract given.

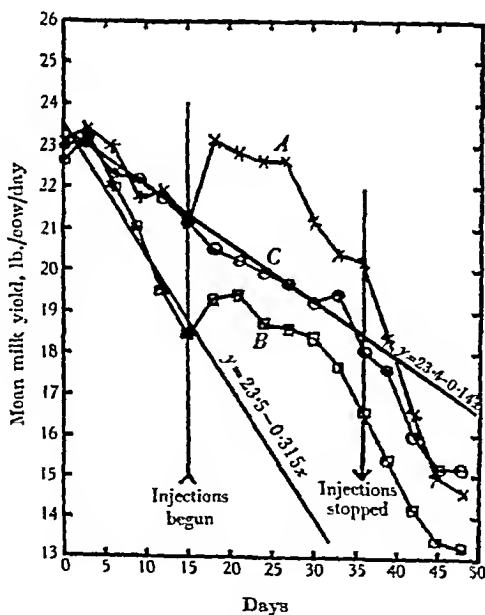


FIG. 2. The influence of different doses of crude anterior-pituitary extract, injected at 2-day intervals for 3 weeks (eleven injections in all) on the milk yields of groups of five cows in declining lactation. The data given are the mean yields for each group for periods of 3 days. Group A, 2.5 g.-equiv. of pituitary extract per injection. Group B, 1.25 g.-equiv. of pituitary extract per injection. Group C, 0.625 g.-equiv. of pituitary extract per injection. The first injection was given, in each case, on day 15, and the last on day 35.  $y = 23.4 - 0.14x$  (where  $y$  = milk yield in lb./cow/day and  $x$  = number of days since control period began) is the best straight line, fitted by the method of least squares, for the pre-injection control data for group A.  $y = 23.5 - 0.315x$  is the corresponding straight line for group B. The similar straight line for group C ( $y = 23.2 - 0.13x$ ) is so similar to that for group A that it has not been included in the figure.

When the injection interval with 2.5 g.-equiv. doses was increased to 3 days (eight injections over a period of 22 days) a substantial increase in milk production was still obtained (Fig. 3), but when the interval was lengthened to 4 days (six injections over a period of 21 days) no observable sustained effect was elicited (Fig. 3).

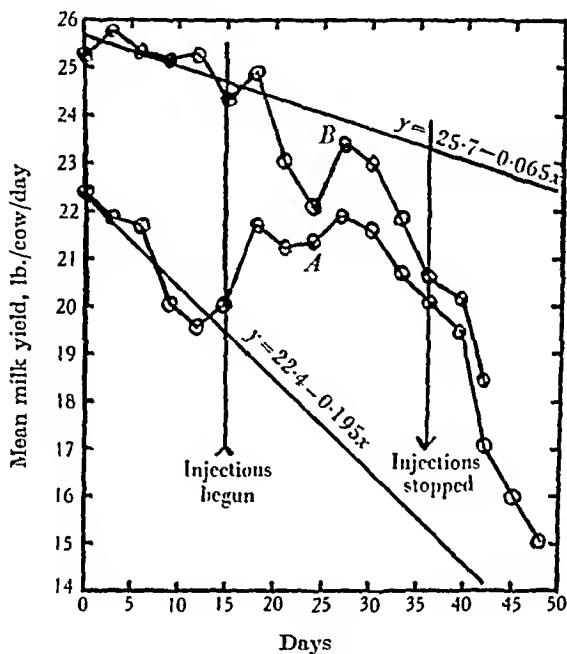


FIG. 3. The influence of doses of 2.5 g.-equiv. of crude anterior-pituitary extract, injected at different intervals for 3 weeks, on the milk yields of groups of cows in declining lactation. The data given are the mean yields for each group for periods of 3 days. Group A, four cows receiving injections at 3-day intervals (eight injections in all). Group B, five cows receiving injections at 4-day intervals (six injections in all). The first injections were given on day 15 and the last on day 36 (group A) or 35 (group B).  $y = 22.4 - 0.195x$  (where  $y$  = milk yield in lb./cow/day and  $x$  = number of days since control period began) is the best straight line, fitted by the method of least squares, for the pre-injection control data for group A.  $y = 25.7 - 0.065x$  is the corresponding straight line for group B.

As the result of these experiments we believed that injections of 2.5 g.-equiv. of crude anterior-lobe extract given at 2-day intervals during 3 weeks (eleven injections in all) might provide a useful and economical means of increasing milk production under the conditions of these preliminary experiments. We then investigated the stability at room temperature of the galactopoietic factors of the crude extract, and the possibility that active extracts might be prepared from fresh whole pituitary glands or from desiccated anterior-lobe tissue.

#### *Activity of stale anterior-lobe extract*

At room temperature the diabetogenic activity of fresh anterior-lobe tissue, or of a saline suspension of the tissue, is rapidly lost; it was therefore important to know whether galactopoietic activity would disappear if a crude alkaline extract of anterior-pituitary tissue were kept at room temperature for 24 hr. It was found (Table 1) that a single injection of 2.5 g.-equiv. of such a stale extract was at least as galactopoietically effective as a similar dose of an extract of fresh tissue. An experiment was

therefore undertaken to determine whether repeated injections at 2-day intervals over a period of 3 weeks of such a stale extract would induce a sustained increase in milk production of the type obtained with fresh extracts (cf. Fig. 2), but unfortunately after this experiment had been in progress for a week the experimental cows, together with their control animals, gained access to an orchard, and as the result of the excessive consumption of apples, scoured very badly. This indisposition resulted in a catastrophic fall in milk production in both the experimental and control groups, though the milk yield later rose as the animals recovered. Throughout the experimental period the pituitary-treated animals gave more milk than the control cows, but the results after the first week were discarded owing to the difficulty of quantitative interpretation. Nevertheless, at the end of the first week of treatment, before the unfortunate incident occurred, the experimental animals were giving 17% more milk than was expected on the basis of the pre-treatment decline in milk production, compared with a mean figure of 16.5% for similar data from four unblemished experiments with fresh anterior-lobe extract. There was therefore no reason to doubt that the stale anterior-lobe extract possessed galactopoietic activity, and indeed it appeared to be just as active as the fresh extract.

*Activity of an extract of whole ox-pituitary gland*

From every point of view there would be great advantage in avoiding the troublesome necessity of dissecting anterior-pituitary tissue free from posterior-lobe material in the preparation of galactopoietic pituitary extract. Folley & Young [1938] found that the addition of posterior-lobe extract to an anterior-lobe preparation did not inhibit the galactopoietic action of a single injection of the latter, and, moreover, there is some evidence that injection of posterior-lobe preparations can retard the rate of decline of milk production in cows [Knodt & Petersen, 1944], but the possibility remained that the repeated administration of whole-pituitary extract over a period of 3 weeks might result in less stimulation of milk production than is obtained with a similar extract of anterior-lobe tissue. Furthermore, as the majority of cows in declining lactation are pregnant, or about to become so, it seemed desirable to determine whether or not any oxytocic effect of whole-pituitary extract might have a deleterious action when pregnant cows received repeated injections of an extract of undissected, whole ox-pituitary gland. It should be pointed out that although the oxytocic posterior-pituitary factor is unstable in alkaline medium we have observed substantial oxytocic activity in an alkaline extract of posterior-lobe tissue prepared in the same way as the galactopoietic factor from anterior-lobe tissue. Table 1 shows the result of an experiment in which a group of four pregnant cows (97, 136, 178 and 182 days pregnant when the pituitary injections began) were given a single subcutaneous injection of an extract of fresh whole ox-pituitary gland, the dose being equivalent to 2.5 g. of fresh anterior-lobe tissue. At the same time a similar group of cows received parallel treatment with the usual extract of ox anterior-lobe tissue. As no control experiment was run simultaneously, it is not possible to indicate the net increase in milk yield resulting from this single injection, but it is clear from the results (Table 1) that the increase was probably similar to those of the previous experiments and that the extract of the whole-pituitary gland certainly gave no smaller effect than did the anterior-lobe extract.



In a further experiment the repeated administration of the pituitary extract at 2-day intervals was continued for 21 days. With respect to milk yield the results, which are not illustrated, were similar in every way to those for comparable treatment with fresh ox anterior-lobe extract (Figs. 2 and 4). Furthermore, no interruption of pregnancy occurred and no subsequent difficulties were reported at parturition.

*Influence of preliminary desiccation of anterior-pituitary tissue on the galactopoietic activity of the crude extract*

If the galactopoietic action of anterior-pituitary tissue were to be utilized on a large scale, desiccation of the tissue for purposes of storage would be very useful. Accordingly, we investigated the influence of desiccation of the fresh tissue with various solvents on the galactopoietic activity of a crude extract.

In preliminary experiments, involving the administration of a single dose of extract equivalent to 2.5 g. of fresh tissue, we found that acetone-desiccated tissue yielded an extract as rich in galactopoietic activity as that from fresh tissue, whereas an extract of tissue which had been desiccated in absolute ethanol, or in methylated spirit, appeared to be inactive under these conditions (Table 2). The reason for the loss of extractable activity on desiccation in ethanol is not clear, though it is possible that alcohol denaturation of protein material may be involved.

Table 2. *Influence of a single subcutaneous injection of an extract of fresh, or of desiccated, anterior-pituitary tissue on the milk yield of cows in declining lactation*

Tissue used for preparation of extract	No. of cows in group	Milk yield			Net increase due to pituitary extract
		Mean for 2 days before injection lb./cow/day	Mean for 2 days after injection lb./cow/day	Increase %	
None	5	22.6	22.9	1.3	—
Fresh undesiccated	2	34.2	38.7	12.9	11.6
Acetone-desiccated	4	26.3	29.9	13.7	12.4
Ethanol-desiccated	4	18.1	18.4	1.7	0.4
'Methcol'*-desiccated	3	22.4	22.0	-1.8	-3.1

10 ml., equivalent to 2.5 g. of fresh anterior-pituitary tissue, was administered to each treated cow.

\* Commercial methylated spirit.

Fig. 4 gives the results of experiments in which doses of extract equivalent to 2.5 g. of fresh anterior-lobe tissue were administered at 2-day intervals over a period of 3 weeks. As was expected from the results of the preliminary experiments, the extract of acetone-desiccated tissue proved to be as effective as that of the fresh undried material, while the extract of the tissue which had been dried in methylated spirit showed little or no significant activity, even when, after 12 days, the dose injected was doubled. A similar experiment, which is not illustrated, was carried out with repeated injection of the extract of tissue which had been dried in absolute ethanol. This extract was found to possess doubtful activity, this being less than one-half of that of the extract of acetone-desiccated tissue. No obviously better result was obtained when the dose of extract of the absolute-ethanol-dried material was doubled.

Figure 1 is a line graph showing the mean milk yield (lb./cow/day) of three Friesian cows (A, B, and C) over 70 days. The y-axis represents mean milk yield in lb./cow/day, ranging from 14 to 44. The x-axis represents days, ranging from 0 to 70. Cow A (top line) starts at approximately 39 lb./day, peaks at about 42 lb./day around day 35, and ends at about 30 lb./day. Cow B (middle line) starts at approximately 30 lb./day, peaks at about 33 lb./day around day 25, and ends at about 26 lb./day. Cow C (bottom line) starts at approximately 26 lb./day, peaks at about 23 lb./day around day 25, and ends at about 18 lb./day. All three cows show a sharp decline in milk yield after day 35. Regression lines are shown for each cow:  $y = 39.1 - 0.18x$  for Cow A,  $y = 29.6 - 0.17x$  for Cow B, and  $y = 26.0 - 0.21x$  for Cow C. Vertical lines indicate 'Injections begun' at day 15, 'Dew doubled' at day 30, and 'Injections stopped' at day 35.

*Total extra yield of milk obtained by means of pituitary treatment*

Table 3. *Total extra milk obtained during experimental and post-experimental periods from cows treated with injections of 2.5 g.-equiv. of anterior-lobe extract*

No. of cows in group	Mean pre-treatment rate of decline in milk yield lb./cow/day/day	Average yield at beginning of treatment lb./cow/day	Total extra milk obtained					
			During experimental period (3 weeks)		During post-experimental period (2 weeks)		During both periods combined (5 weeks)	
			lb./cow	% of that expected in absence of treatment	lb./cow	% of that expected in absence of treatment	lb./cow	% of that expected in absence of treatment
2	0.18	34.2	119.4	16.5	95.3	20.6	215.7	18.1
4	0.17	26.3	144.4	27.4	51.0	15.3	195.4	22.6
5	0.14	21.2	44.1	10.6	7.3	8.5	36.8	7.3
4	0.05	9.5	44.1	25.0	6.0	5.3	38.1	13.2
4	0.11	8.0	56.4	45.2	45.6	76.8	102.0	55.5
Weighted mean	0.125	18.4	75.6	21.0	27.3	15.3	103.0*	19.1

- Equivalent to about 10 gallons.

yield actually obtained during the experimental and post-experimental periods. Control groups of cows were treated with thymus extract during the experimental period and showed the uninterrupted decline in milk yield predicted from the pre-injection data. These control data, which are not given in detail, support the validity of our method for determining the extra milk produced as the result of pituitary treatment.

Although substantial differences were observed between the results in the different groups of animals, these variations could not obviously be related to differences in the average yield at the beginning of treatment, nor to differences in the mean rate of decline in milk yield during the pre-treatment period. On the average the extra milk obtained during the whole period of 5 weeks (experimental plus post-experimental periods) was 103 lb./cow or approximately 10 gal./cow. In the absence of treatment, the animals should have yielded, on the average, about 50 gal./cow during this period, so we can say in round figures that during the 5-week period the nineteen cows gave an average of 60 gal. of milk per cow instead of an expected 50 gal. With respect to milk production of cows in declining lactation, it would seem reasonable to regard this group of nineteen cows as being in a condition representative of the average for good-class herds in this country, and if a comparable increase could be effected in similar herds over the whole country it clearly would provide a valuable source of extra milk.

Table 3 shows that in two of the groups less milk was obtained during the 2 weeks following cessation of pituitary treatment than was expected from the pre-treatment rate of decline. On the other hand, for three of the other groups the yield was substantially greater, during the post-injection period, than would be expected in the absence of treatment. In the first two groups, part of the available data for which is illustrated in Fig. 4 (curves *A* and *B*), the milk yield appeared to be sustainedly enhanced as the result of the pituitary treatment, and even 4 months after the beginning of the experiment was still constantly more than 2 lb./day above the extrapolated 'expected' curve. Although the results of such an extensive extrapolation may well be unreliable, it seems probable that the milk yield was at any rate not subsequently depressed as the result of treatment in these two cases. In the two groups in which the yield during the 2-week post-injection period was below that expected, the depression appeared to be only temporary and no obvious general diminution in the expected milk supply was observed. We therefore feel justified in the assumption that as the result of pituitary treatment, the average extra amount of milk obtained over a period of 5 weeks, from cows which would yield 50 gal. of milk during this time in the absence of treatment, was 10 gal./cow, or 20 %.

#### DISCUSSION

The results of this investigation show that, under the conditions of our experiments, cows in declining lactation can be induced to yield over 20 % more milk than would otherwise be expected, when they are treated with anterior-pituitary extract for a period of 3 weeks. The increase is sustained for a time at least in that the yield for 2 weeks immediately following the injection period is 15 % above that expected (Table 3). This result was obtained when 2.5 g.-equiv. of fresh ox anterior-pituitary extract was administered subcutaneously to the cows on every second day for 3 weeks, eleven injections being given in all.

The loss of extractable activity produced by ethanol desiccation of anterior-lobe tissue may result from inactivation of the galactopoietic complex by the ethanol, or it may follow a diminution in aqueous solubility of the complex, brought about by ethanol desiccation of the tissue proteins, etc. Which of these possibilities is relevant we cannot say, but it is interesting to note that since prolactin is not destroyed or rendered insoluble by desiccation of the tissue in ethanol this observation agrees with our previous findings [Folley & Young, 1938, 1939, 1940, 1941] that prolactin in itself is probably not galactopoietic, though it may constitute one component of a galactopoietic complex.

As the result of experiments in which different doses of fresh anterior-lobe extract were administered every second day for 3 weeks, and experiments in which a dose of fresh extract equivalent to 2.5 g. of anterior-lobe tissue was administered every third or every fourth day for the same period, it was concluded that the administration of 2.5 g.-equiv. every second day for 3 weeks provided a means of ensuring an adequate galactopoietic stimulus with economical use of the available extract. The results of field trials in which this technique was employed will be published later.

Although systematic analyses of the milk were not made in the present experiments, our previous results have shown that little or no change in the composition of milk results from pituitary stimulation of milk production in cows in declining lactation [Folley & Young, 1938, 1939], though there is a tendency for the milk-fat content to rise [cf. also Asimov & Krouze, 1937; Sykes, Meuleman & Huffman, 1942].

The question of the nature and identity of the galactopoietic complex of the anterior-pituitary gland is not one we wish to discuss in the present context, but it may be stated that our present results agree with our published opinions on the subject [cf. Folley & Young, 1941].

The cost of the ox-pituitary glands obtained for this experimental work was 6d. per gland. On this basis the expense of treatment over 3 weeks was 11s. per cow. The extra milk obtained (10 gal./cow—Table 3) can be regarded as bringing a profit to the farmer of 1s. 6d. per gal., which means a total gross profit of 15s. The net profit per cow is therefore 15s. minus 11s., which is 4s. The cost of the pituitary glands, and therefore of treatment, could undoubtedly be substantially reduced if the process were carried out on a large scale, and there seems no doubt that, if all cows reacted similarly, financially it would be highly profitable to the individual dairy farmer who wished to increase his total milk production. The cows in these experiments were fed according to accepted standards of first-class management, which means that the ration given to the animal varies according to its milk yield. The above financial calculations are based on a figure for the profit on extra milk which takes account of the extra food necessitated by an increase in milk production. But the cows were not weighed during the experiments nor were observations recorded with regard to heart rate and body temperature, so that we cannot say whether or not the treatment caused any change in body weight or metabolic rate. It can be stated, however, that the expert herdsmen in charge of the cattle noted no obvious losses in 'condition' resulting from treatment. The question of whether or not pituitary treatment increases the efficiency of conversion of foodstuffs to milk is not therefore one which we can discuss here, although some relevant experiments will be reported later.

If the process of stimulation of milk production by pituitary treatment were to be used on a nation-wide scale, consideration would have to be given to the total supplies

of anterior-pituitary material continuously available in this country, and to the fractional increase in the nation's milk supply which could be induced, under favourable circumstances, by the treatment. It seems improbable that more than 20,000 g. of ox anterior-pituitary tissue could be collected each week in this country, which would allow of the treatment of about 2,200 cows for a 3-week period. Thus about 38,000 cows could be treated each year, with an extra milk yield of about 380,000 gal. This is certainly less than 0.05 % of the total fresh milk supply of the country, and, even if treatment with all the available pituitary extract were given during the winter months only, it is clear that no significant increase in the nation's milk supply would accrue from the employment of pituitary stimulation as a routine process. Nevertheless, this does not detract from the fact that to the individual farmer the successful use of this process might be of considerable financial advantage.

#### SUMMARY

1. When a dose of crude extract of fresh ox anterior-pituitary tissue, equivalent to 2.5 g. of fresh gland, was subcutaneously administered to cows in declining lactation every second day for 3 weeks, the milk production during the period of treatment was, on the average, more than 20 % above that expected in the absence of treatment. During the 2 weeks immediately following treatment the yield was 15 % above that expected.

2. When smaller doses of extract were used, or if the extract was administered at intervals of 3 or of 4 days, the increase in milk yield was less certain.

3. The relation between the galactopoietic response to single injections of extract and the dose of extract administered is expressed by a sigmoid curve.

4. An extract prepared from whole ox-pituitary gland, an extract (of fresh anterior-lobe tissue) which had been allowed to remain at room temperature for 24 hr., and an extract of acetone-desiccated anterior-lobe tissue, were all not obviously less galactopoietic than our standard extract of fresh ox anterior-lobe tissue. Extracts of ethanol-desiccated anterior-pituitary tissue were almost inactive.

5. Repeated injections of an extract prepared from whole ox-pituitary gland had no deleterious effect on pregnant cows.

We wish to record our gratitude for the generous and willing co-operation of the late Major G. W. Dunkin, Mr S. J. Edwards and Mr E. A. Macmillan, of the Agricultural Research Council's Field Station, Compton, Berks; of Dr W. K. Slater, Mr H. T. Fawns and Mr F. U. Crook of Dartington Hall, Totnes, South Devon; and of Prof. R. Rae and Mr K. W. D. Campbell of the University of Reading Farm. The cost of these investigations was defrayed by special grants from the Agricultural Research Council and from the Medical Research Council, to whom we wish to express our thanks.

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# THE GALACTOPOIETIC ACTION OF PITUITARY EXTRACTS IN LACTATING COWS

## 2. THE RESPONSE DURING THE PEAK OF LACTATION

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(Received 13 October 1944)

In the previous paper [Folley & Young, 1945] we showed that cows of the breeds we have employed and under the conditions of our experiments respond regularly to the administration of anterior-pituitary extract during the period of declining lactation, an increase in total milk yield of about 20% above that expected in the absence of treatment being obtained when a suitable dose was administered at 2-day intervals over a period of 3 weeks. Although during the 2 weeks immediately following cessation of treatment the mean yield was still above that expected, the effect was not sustained throughout the period of lactation and the daily yield ultimately became similar to that which might have been expected had pituitary treatment not been instituted.

It seemed to us possible that if, instead of cows in declining lactation, animals before or during the peak of lactation were similarly treated, it might prove possible to raise and prolong the peak of milk production. If, in such a case, the subsequent decline occurred at a rate which was to be expected in the absence of injections, then the treatment would have exerted a galactopoietic effect covering the whole of the lactation cycle and resulting in a substantial overall increase in milk production.

In discussing the influence of pituitary extracts in increasing the milk yield of lactating animals, Petersen states in a recent review, 'In all experiments of this kind it has been found that lactation increases are greatest when treatment is made in the declining phase of lactation' [Petersen, 1944]. Asdell, Brooks, Salisbury & Seidenstein [1936] found that treatment of milking goats with prolactin had no influence on milk production if treatment was begun just after the peak of lactation, but when similar treatment was started later in the lactation period the milk yield was definitely increased. As the result of experiments with 510 cows in various stages of lactation, ranging from 1 to 21 months after calving, Asimov & Krouze [1937] conclude that 'the injection of the lactogenic substances is more effective during the first half of lactation (2-6 months)'. This, by omission, seems to imply that treatment was less effective during the first month after calving, but in the absence of data regarding the time of occurrence of the peak of lactation in these animals it is difficult to determine whether or not Asimov & Krouze found any galactopoietic effect when their pituitary extract was administered at the peak.

Although all these results were not encouraging, it nevertheless seemed desirable to determine whether cows of known reactivity to the galactopoietic action of our extract during declining lactation would react at the peak of lactation.

## METHODS

*Animals*

A total of fifteen cows at or close to the peak of lactation have been used in these experiments. They were owned by Dartington Hall Ltd., and were of the South Devon breed, which we have shown to be very responsive, during declining lactation, to the galactopoietic action of our crude anterior-pituitary extract.

*Anterior-pituitary extracts*

We used either our standard crude alkaline extract of fresh ox anterior-pituitary tissue, or a similar extract of acetone-desiccated ox anterior lobe [Folley & Young, 1945]. In every case the material extracted from 2.5 g. of fresh anterior-lobe tissue or 0.5 g. of desiccated gland was contained in 10 ml. of extract.

*Treatment of animals*

The dose used throughout these experiments was 10 ml. of standard extract, equivalent to 2.5 g. of fresh ox anterior-lobe tissue. It was injected subcutaneously at 2-day intervals for varying periods, the injections usually being given before or during the afternoon milking period. All animals were milked twice daily.

## RESULTS

*Control observations*

The interpretation of the results of experiments on animals at the peak of lactation is beset by the difficulty of obtaining satisfactory control data for any particular animal. With this difficulty in mind we constructed a mean lactation curve (Fig. 1, curve A), over a period of 5 months after calving, of cows of the type we have used, based on the data for seven animals in a condition similar to that of the experimental animals. The results (Fig. 1, curve A) suggest that, under the conditions of our experiments, the animals remain at a level of milk production which is maximal, or nearly maximal, for a period of about 5 weeks, lasting from 2 weeks until about 7 weeks after calving. Thereafter the decline in milk production is fairly steady for a further period of about 7 weeks, after which the rate of fall diminishes.

As the result of eight previous experiments with the herd of Dartington Hall Ltd., we had observed a mean rate of decline of  $0.194 \pm 0.008$  lb./cow/day/day (mean and standard deviation of the mean) for the early part of declining lactation (yield 30 lb./day or above) and a slope of  $0.074 \pm 0.008$  lb./cow/day/day for the later part of the curve of decline (yield less than 20 lb./cow/day). The slope for our present control curve (Fig. 1) for the same herd is approximately 0.18 lb./cow/day/day for 7–14 weeks after calving, and 0.10 lb./cow/day/day for 14–21 weeks after calving (Table 1).

*Influence of a single injection of pituitary extract*

We have shown [Folley & Young, 1945] that when a single injection of 2.5 g.-equiv. of anterior-pituitary extract is given to cows in declining lactation the milk yield rises, during the 2 days immediately following the injection, 7–10% above the mean yield for the 2 days immediately preceding the injection. By contrast, cows in the

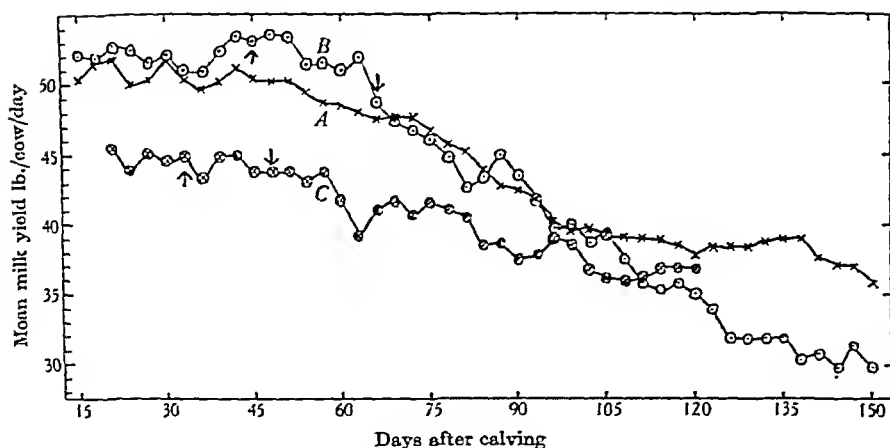


FIG. 1. Influence of repeated injections of anterior-pituitary extract on the milk production of cows at the peak of lactation. Curve A, mean curve for control animals based on data for seven cows similar to those under the experimental treatment and living under the same conditions. Curve B, data for one animal (Jill) treated for 3 weeks, beginning 45 days after calving, with 2.5 g.-equiv. of fresh pituitary extract on alternate days. Curve C, mean data for two animals (Bradley 2 and Primrose 1) treated for 2 weeks, beginning 48 days after calving, with 2.5 g.-equiv. of extract of acetone-desiccated pituitary gland on alternate days. The data plotted are the mean yields for 3-day periods.

Table 1. Rates of decline of milk production in different experiments

Group	Approximate rate of decline in milk production (lb./cow/day/day) over period	
	7-14 weeks after calving	14-21 weeks after calving
A, Control (Fig. 1)	0.18	0.10
B (Fig. 1)	0.27	0.25
C (Fig. 1)	0.11	0.11
D (Fig. 2)	—	0.37
E (Fig. 3)	0.18	—
F (Fig. 4)	0.30	—
G (Fig. 4)	0.19	—

7 weeks after calving during which milk production is approximately constant, showed no rise in two experiments in which a single dose of pituitary extract was administered 33 and 46 days respectively after calving (Table 2). In a third experiment the treated animal had calved 10 weeks previously, so that it had, according to our control data above, entered the first phase of diminution in milk production and might therefore be expected to react like a cow in declining lactation. This expectation was fulfilled in that the single injection of anterior-lobe extract elicited an increase in milk yield of 10.3% over the 2-day period (Table 2).

These preliminary experiments suggested that milk production in the cow, like that in the goat [Asdell *et al.* 1936], might prove to be less responsive to pituitary stimulation during the peak of lactation than during the period of declining production. Experiments involving the repeated administration of pituitary extract were then undertaken in order further to investigate this possibility.



Table 2. *Influence of a single injection of 2.5 g.-equiv. of ox anterior-pituitary extract on the milk yield of cows at the peak of lactation*

Group	No. of cows in group	Days after calving	Source of pituitary extract	Milk yield			Net increase (pituitary minus control) %
				Mean for 2 days before injection lb./cow/day	Mean for 2 days after injection lb./cow/day	Increase %	
Pituitary	2	33	Acetone-dried tissue	44.9	42.7	-4.9	
Control	2	—	—	51.2	49.7	-2.9	- 2.0
Pituitary	1	46	Fresh tissue	53.8	53.6	-0.4	
Control	1	—	—	24.9	26.2	+5.2	- 5.6
Pituitary	1	69	Fresh tissue	41.2	43.7	+6.1	
Control	1	—	—	28.4	27.2	-4.2	+10.3
Pituitary	1	18* (abortion)	Fresh tissue	47.2	50.5	+7.0	
Control	1	—	—	28.4	27.2	-4.2	+11.2

\* Calved about 9 weeks prematurely.

#### *Influence of repeated injection of anterior-pituitary extract*

*Treatment during post-calving period.* Fig. 1 gives in graphical form the results of the two experiments in which cows were given repeated injections of anterior-pituitary extract at 2-day intervals during the 7 weeks of steady milk production following calving. No galactopoietic action of the anterior-pituitary extract was in evidence (Fig. 1) and the subsequent rate of decline in milk production was in one case (*B*, Fig. 1 and Table 1) possibly significantly greater than that of the control group, though it would be unsafe to make a firm deduction on this point from the results of one experiment. The rate of decline of milk production in the other group of treated animals (two cows—*C*, Fig. 1 and Table 1) was perhaps slightly less than that of the controls over the first period of declining lactation.

When a cow received treatment for 3 weeks beginning 10 weeks after calving, when the first phase of declining lactation had begun in our control animals, a significant galactopoietic response was observed (Fig. 2). This, however, was less than we have found with comparable animals similarly treated later in the period of declining lactation, the aggregate increase in milk production during the period of treatment being only 8.1 % above that expected. This increase is less than half that found with cows later in declining lactation [Folley & Young, 1945]. Furthermore, the rate of decline in milk production following treatment was significantly greater than that in the control group, being 0.37 lb./cow/day/day for the period 14–21 weeks after calving, compared with the control figure of 0.10 lb./cow/day/day (Fig. 1 and Table 1).

*Treatment before calving.* Two cows were treated with pituitary extract for about 5 weeks before calving and for 3 weeks after. The mean result for these two animals (Fig. 3) suggested the possibility that the maximum yield had been increased slightly for the early part of the post-calving period of steady milk production. Between 5 and 7 weeks after calving the milk yield declined rapidly until the control level had been reached, after which the rate of fall for the first phase of the period of declining lactation was similar to that for the control group (0.18 lb./cow/day/day—

Table 1). Thus the extra milk produced as the result of the prolonged pituitary treatment could not have been substantial even if it were significant.

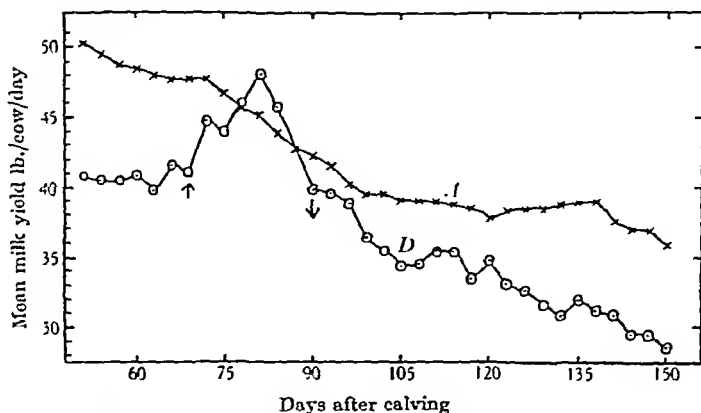


FIG. 2. Influence of repeated injections of anterior-pituitary extract on milk production at the end of the peak of lactation. Curve A, mean data for seven control animals. Curve D, data for May 7 which received treatment with extract of fresh gland over the period 69-90 days after calving. Doses of 2.5 g.-equiv. were given on alternate days over this period. The data plotted are the mean yields for 3-day periods.

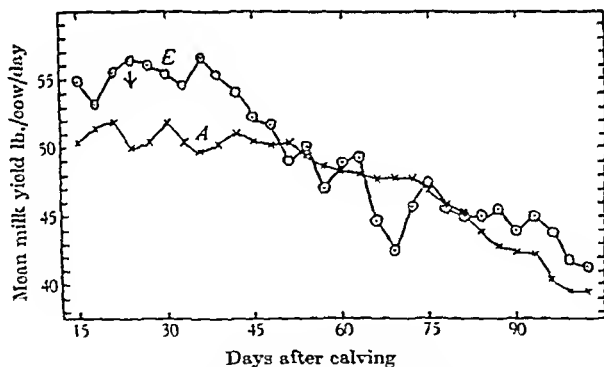


FIG. 3. Influence on milk production of repeated injections of anterior-pituitary extract before calving. Curve A, mean data for seven control animals. Curve E, data for two animals (Crocus 2 and Milkmaid 34) which received treatment with an extract of acetone-desiccated anterior-pituitary tissue for 5 weeks before calving and for 3 weeks after. During this period doses of 2.5 g.-equiv. were given on alternate days. The data plotted are the mean yields for 3-day periods.

*Treatment, during the post-partum period, of cows which had aborted.* The disappointing results of the treatment of normal cows at the peak of lactation suggested that the rate of liberation of anterior-lobe secretions was not the limiting factor, or at least not the sole limiting factor, in determining the rate of secretion of milk during the first 7 weeks after calving. The possibility remained, however, that in the special case of the cow which has aborted and which therefore is producing less milk at the peak of lactation than otherwise might have been expected, the rate of liberation of pituitary secretions is subnormal and has become the limiting factor in determining the rate of milk production during the period immediately after birth. If this were

so, then such animals might exhibit a galactopoietic response to the administration of pituitary extract shortly after calving. This expectation was realized in experiments on two cows, the data for which are given in Fig. 4. Calculation showed that, as the result of pituitary treatment for 3 weeks beginning 18 days after parturition, Daisy 5 (curve *F*, Fig. 4), during the period of injection, gave 11.6% more milk than was expected in the absence of treatment. Although this is less than that generally

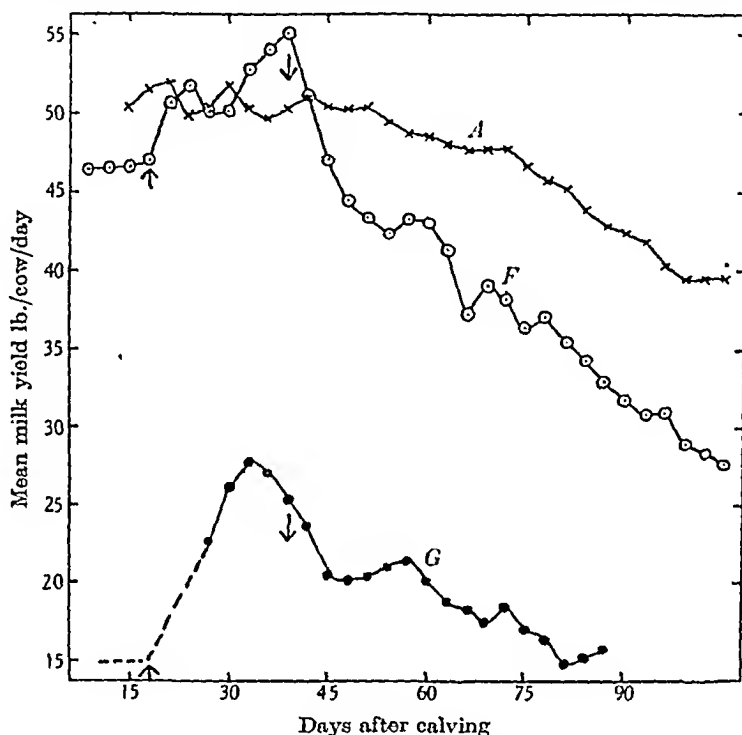


FIG. 4. Influence of pituitary treatment, instituted within 3 weeks of parturition, on the milk production of cows which had aborted. Curve *A*, mean data for seven control animals. Curve *F*, data for Daisy 5 which calved about 8 weeks prematurely. This animal was given injections, at intervals of 2 days, of 2.5 g.-equiv. of fresh pituitary extract, over the period 18–39 days after calving. Curve *G*, data for Pansy, which calved about 9 weeks prematurely and was treated similarly to Daisy 5 (curve *F* above). The data plotted are the mean yields for 3-day periods.

given by similar cows in declining lactation, it is nevertheless significant. A similar calculation could not be made on the data for Pansy (curve *G*, Fig. 4), owing to the fact that for  $3\frac{1}{2}$  weeks after abortion she was suckling calves.

It is a common experience that cows which have aborted dry off more quickly than usual. The rate of decline in the milk production of Daisy 5 over the period 7–14 weeks after calving was 0.30 lb./cow/day/day (Table 1), probably significantly greater than the corresponding figure for the control group (0.18, Table 1). Whether it would have been still greater in the absence of pituitary treatment cannot be determined. The rate of decline of milk production for Pansy (curve *G*, Fig. 4) over a similar period was 0.19 lb./cow/day/day which is similar to the control figure.

#### DISCUSSION

The results clearly show that the milk yield from these cows is not increased by injections of anterior-pituitary extract during the peak of lactation which is present

for the first 7 weeks after calving and that they therefore differ from similar cows in declining lactation when a substantial rise in milk production may be consistently produced by such treatment [Folley & Young, 1945]. As we suggested above, it is possible that during the peak of lactation the factor limiting the production of milk is not solely the rate of liberation of anterior-pituitary secretions. If this is so, the enhancement of such secretions, or their artificial enrichment by the introduction of an exogenous ox anterior-pituitary preparation, would not be expected to increase the milk production of the animals at this stage. If one cause of the decline in milk secretion after the 7 weeks or so of steady production is a diminution of the rate of secretion of the relevant anterior-pituitary factors, then it is not surprising that after the peak period is past the animals become sensitive to the galactopoietic action of injected anterior-lobe preparations.

Again, if these ideas are correct, the diminished milk production by the animal which has aborted her calf results from a subnormal pituitary secretion, since in such a case the administration of anterior-pituitary extract entails the expected galactopoietic effect.

Whatever be the endocrinological significance of our results, it is clear that any large-scale stimulation of milk production by the administration of anterior-pituitary extract should be carried out with animals in declining lactation, i.e. more than 7-10 weeks after calving. The hopes expressed in the introduction to this paper have thus been unfulfilled.

#### SUMMARY

1. In the cows used in the present investigation milk production remains at a steady high level for about 7 weeks after calving and thereafter declines. Normal animals do not respond to the galactopoietic action of anterior-pituitary extract during this period of steady milk production, in this respect differing from similar cows in the period of declining lactation.

2. Two cows which had aborted their calves did respond to the galactopoietic influence of pituitary treatment given within 3 weeks of parturition.

3. The possibility is considered that during the normal period of steady milk production after calving the factor limiting the output of milk is not the rate of liberation of endogenous anterior-pituitary secretion, though this endocrine factor may be a limiting one in animals which have aborted and in those which have reached the normal period of declining lactation.

We wish to record our gratitude to Dr W. K. Slater, Mr J. B. E. Patterson and Mr F. U. Crook, of Dartington Hall, Totnes, South Devon, for their interest and co-operation in this work. The cost of this investigation was defrayed by special grants to two of us (S. J. F. and F. G. Y.) from the Agricultural Research Council and from the Medical Research Council, to whom we wish to express our thanks.

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# THE GALACTOPOIETIC ACTION OF PITUITARY EXTRACTS IN LACTATING COWS

## 3. COMPARISON OF EXTRACTS OF PITUITARY GLANDS FROM DIFFERENT SPECIES

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(Received 13 October 1944)

In the first paper of this series we showed [Folley & Young, 1945*a*] that when cows in declining lactation were treated with ox anterior-pituitary extract at 2-day intervals over a period of 3 weeks, a substantial increase in milk production was evoked, amounting to more than 20% above that which was expected in the absence of treatment. It was calculated, however, that even if all the ox anterior-pituitary tissue available in Great Britain was utilized for the purpose of increasing the nation's milk supply, only a negligible increase could thus be effected. We therefore decided to investigate the activity of pituitary glands from different species of animal in the hope that greater activity might be found in sheep, horse or pig pituitary gland than had previously been observed for ox tissue. Moreover, the opportunity was taken of testing the activity of material imported into this country in a desiccated condition from the United States. If by chance such material were highly active, it might be used to supplement the limited supplies available in this country.

Although many observations have been recorded regarding species differences in the amounts of the various hormones present in the anterior-pituitary lobe [cf. Chance, Rowlands & Young, 1939], we have published evidence [cf. Folley & Young, 1938, 1941] that the so-called lactogenic hormone is not the only pituitary factor concerned in galactopoiesis in milking cows. Such being the case, it seemed that nothing could justifiably be hazarded *a priori* about the relative galactopoietic effects of extracts prepared from the pituitary glands of different species.

### METHODS

#### *Animals*

These were twenty-four Shorthorn cows in declining lactation belonging to the herd of Viscount Astor at White Place Farm, Cookham, Berkshire. They were divided into six groups of four animals for the purposes of the experiments, the animals in the various groups being remarkably uniform with respect to milk yield, time since mating, etc. The cows were milked twice daily.

#### *Anterior-pituitary extracts*

All the extracts used in the present experiments were prepared from acetone-desiccated anterior-lobe tissue by extraction with saline at pH 8.5 [cf. Folley & Young, 1945*a*]. In every case the final concentration was such that 10 ml. contained the material which had been extracted from 0.5 g. of the acetone-desiccated material, this amount being taken as equivalent to 2.5 g. of fresh gland.

In these experiments we used two types of acetone-desiccated ox pituitary gland. One we prepared ourselves [cf. Folley & Young, 1945a] from absolutely fresh ox anterior lobes, and stored it at room temperature for some time; the other was a sample of commercial desiccated ox anterior-pituitary gland obtained, through the courtesy of Dr S. B. Bradshaw and Dr A. S. Parkes, from Armour Laboratories, Chicago, Ill., U.S.A. The preparations of desiccated sheep and pig anterior-pituitary lobe were obtained similarly from Armour Laboratories. The acetone-desiccated horse pituitary gland was collected in different *abattoirs* in this country where the glands were removed as soon as possible after the death of the animal and immersed in absolute acetone. They were stored in acetone until brought to the laboratory after periods varying from a few days to several weeks. In the laboratory the glands were dissected and the desiccation completed in the usual way [Folley & Young, 1945a].

### *Treatment of animals*

After the usual control period the cows in four groups were given seven injections of 10 ml. (2.5 g.-equiv.) of pituitary (ox, sheep, or pig) extract at 2-day intervals followed by two similarly spaced injections of 20 ml. of extract (nine injections in all during 17 days); the animals in a fifth group were treated similarly with saline instead of anterior-pituitary extract, while the animals in the remaining group of four were given a single injection of horse pituitary extract.

The injections were made subcutaneously during the afternoon milking period.

## RESULTS

### *Influence of a single injection of pituitary extract*

In preliminary experiments a single injection of extract was made into the various groups of cows, and the influence on milk production assessed in terms of the mean percentage increase during the 2 days immediately following the injection as compared with the mean level for the 2 days preceding the injection [cf. Folley & Young, 1945a]. With an extract of fresh or of acetone-desiccated ox anterior-pituitary lobe an increase of 7–10% is usually observed. As we had not previously carried out tests on the present herd of animals, it seemed desirable to include in these experiments a test with a pituitary extract which, from experiments with animals of known reactivity, we knew to be active. Accordingly we included a test with a sample of an extract of the acetone-desiccated ox pituitary tissue which had been shown to be highly effective in previous experiments on galactopoietic activity [Folley & Young, 1945a]. The results (Table 1) showed that the animals of this herd appeared to be somewhat less sensitive to the galactopoietic action of a single dose of the extract of our own desiccated ox gland under these conditions than were the animals we had previously used; nevertheless, the observed net rise of 6.2% was definite and significant. The commercial acetone-desiccated ox gland appeared to be rather less active than our own material. The extracts of the sheep and pig gland appeared to be only poorly active, but the horse pituitary extract possessed high galactopoietic activity according to the results of this preliminary test, possibly greater than that of the ox material.

Table 1. *Influence of a single subcutaneous injection of 2.5 g.-equiv. of extract of pituitary glands from different species on the milk yield of cows in declining lactation*

Extract	No. of cows in group	Milk yield			Net increase (pituitary minus control) %
		Mean for 2 days before injection lb./cow/day	Mean for 2 days after injection lb./cow/day	Change %	
Saline (control)	4	25.6	24.9	-2.9	—
Ox gland	4	27.3	28.2	+3.3	6.2
Ox gland (commercial)	4	26.2	26.7	+1.8	4.7
Horse gland	4	20.7	22.2	+7.5	10.4
Sheep gland	4	26.6	26.4	-0.8	2.1
Pig gland	4	27.7	27.5	-0.7	2.2

In the circumstances it appeared tempting to carry out a more prolonged test with the horse pituitary gland, but consideration of the high gonadotrophic (follicle-stimulating) activity of horse pituitary gland compared with that of the other species under test [cf. Chance *et al.* 1939] led us to abandon this project because of the danger of inducing the formation of cystic follicles in the ovaries of the test animals, with subsequent interference with their reproductive capacity. For these reasons further tests were limited to those with the extracts of ox, sheep and pig pituitary glands.

#### *Influence of repeated injection of pituitary extract*

The changes in milk production resulting from repeated injections of ox, sheep or pig pituitary extracts are compared, in Fig. 1, with those in the control group treated similarly with saline. It will be seen that after about a week of treatment the milk yields of the groups treated with sheep and pig pituitary extracts sharply diminished, and continued to do so for some days after treatment was stopped. The animals receiving the ox pituitary extract exhibited a definite rise in milk production, though a much smaller one than was obtained in previous experiments on other herds of cows with the same dose of extract [Folley & Young, 1945*a*].

In Table 2 are given the figures for the extra milk obtained during the experimental period and for 2 weeks after. The gross extra milk obtained was computed from the difference between the yield expected in the absence of treatment, as determined by extrapolation of the best straight line fitting the data for a pre-treatment period of about 2 weeks, and the yield actually obtained during the experimental and post-experimental periods. When this procedure was applied to the data for the control group of cows, which received treatment with saline, it was found that rather less milk was actually obtained during the experimental and post-experimental periods than was expected on the basis of the pre-treatment rate of decline in milk production. Accordingly the net extra milk yields for the pituitary-treated groups have been calculated by correcting for this deviation in the control group (Table 2).

The data show that the amount of extra milk obtained from the animals treated with ox pituitary extract was less than one-half of that obtained in our previous experiments carried out on other herds [Folley & Young, 1945*a*], notwithstanding the fact that in the present experiments the dose was doubled for the last two injections. This discrepancy does not detract, however, from the significance of the

finding that the cows treated with sheep and pig pituitary extracts produced much less milk than would have been expected in the absence of treatment, this effect persisting for some weeks after stopping treatment (Fig. 1 and Table 2). This fall in milk production was not accompanied by any signs of ill-health or discomfort on the part of cows under treatment, and there seemed no reason to doubt that it was

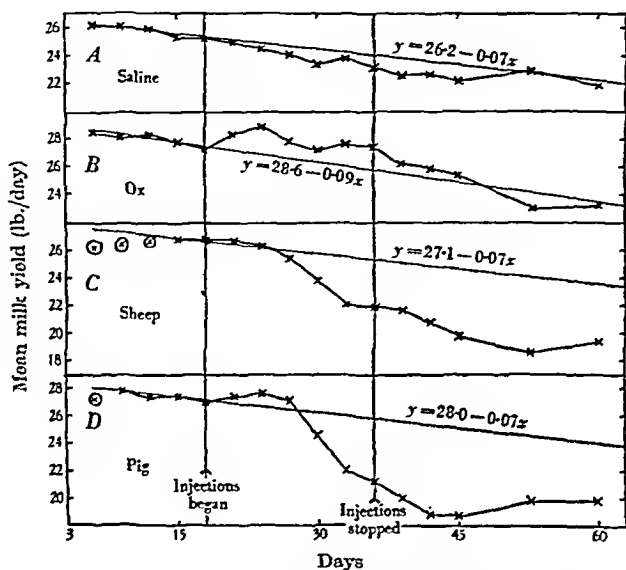


FIG. 1. The influence of repeated injections of different pituitary extracts on the milk production of groups of four cows in declining lactation. In every case seven injections of 10 ml. of extract, equivalent to 2.5 g. of fresh tissue (approx.), followed by two injections of 20 ml. were administered at 2-day intervals, nine doses in all being administered during 17 days. The first injection was given on day 18 and the last on day 34. The data plotted are the mean milk yields for 3-day periods. A, control group (saline). B, extract of acetone-desiccated ox anterior lobe. C, extract of acetone-desiccated sheep anterior lobe. D, extract of acetone-desiccated pig anterior lobe. For each experiment the straight line drawn is that fitted to the pre-injection data by the method of least squares. The equations of these straight lines are of the form  $y = ax - b$ , where  $y$  = mean milk yield in lb./day and  $x$  = time in days since the milk records began. In fitting the straight lines the points ringed in the figure were disregarded. It should be noted that in the case of the group receiving sheep pituitary extract, the milk yield of which behaved anomalously during the greater part of the pre-injection period, in order to obtain some idea of the post-injection decrease in yield below that expected in the absence of treatment, a straight line parallel to that calculated for the control group was drawn through the last two points falling in the pre-injection period.

indeed the result of a physiological or pharmacological effect of the pituitary extract. In this connexion it is of interest to note that for the first week of treatment the milk production of the group treated with pig pituitary extract rose slightly, while the group receiving sheep pituitary extract at least exhibited no sharp decline in milk production until the end of the first week of treatment. This is additional evidence that the effect of the treatment was not a non-specific one affecting adversely the general health and well-being of the animals.



Table 2. *Total extra milk obtained during 17 days' treatment with the different anterior-pituitary extracts*

Extract	No. of cows in group	Mean pre-treatment rate of decline in milk yield lb./cow/day/day	Average yield at beginning of treatment lb./cow/day	Total extra milk obtained		
				Gross		Not (pituitary minus control)
				lb./cow	% of that expected in the absence of treatment	% of that expected in the absence of treatment
During experimental period (2½ weeks)						
Saline (control)	4	0.07	25.3	- 11.1	- 2.5	—
Ox gland	4	0.09	26.9	+ 23.7	+ 5.0	+ 7.5
Ox gland (commercial)	4	0.11	26.0	+ 15.9	+ 3.6	+ 6.1
Sheep gland	4	0.07	26.6	- 30.9	- 6.7	- 4.2
Pig gland	4	0.075	27.2	- 24.0	- 5.1	- 2.6
During post-experimental period (2 weeks)						
Saline (control)	4	0.07	25.3	- 12.9	- 3.6	—
Ox gland	4	0.09	26.9	+ 1.2	+ 0.3	+ 3.9
Ox gland (commercial)	4	0.11	26.0	+ 33.6	+ 10.0	+ 13.6
Sheep gland	4	0.07	26.6	- 77.4	- 20.4	- 16.8
Pig gland	4	0.075	27.2	- 87.0	- 23.1	- 19.5
During both periods combined (4½ weeks)						
Saline (control)	4	0.07	25.3	- 24.0	- 3.0	—
Ox gland	4	0.09	26.9	+ 24.9	+ 2.9	+ 5.9
Ox gland (commercial)	4	0.11	26.0	+ 49.5	+ 6.2	+ 9.2
Sheep gland	4	0.07	26.6	- 108.3	- 12.8	- 9.8
Pig gland	4	0.075	27.2	- 111.0	- 13.0	- 10.0

## DISCUSSION

The results of the experiment in which the influence on milk production of a single injection of pituitary extract was determined showed that horse hypophysis was at least as active as ox gland in this respect if not considerably more so (Table 1), while sheep and pig pituitary extracts exhibited little or no activity. These results cannot be correlated with the relative prolactin contents of the pituitary glands from the different species, for horse tissue is relatively deficient in prolactin [Chance *et al.* 1939] while sheep pituitary is one of the richest sources of this substance. Thus we may emphasize once again our contention that prolactin is by no means the only factor, nor necessarily the most important factor, concerned with the galactopoietic action of anterior-pituitary extracts. Consideration of the hormonal patterns of the pituitary glands of the species under discussion as determined by Chance *et al.* [1939], gives little aid in accounting for the relative galactopoietic activities demonstrated in our preliminary result (Table 1). Horse pituitary extract is characterized by a relatively high content of follicle-stimulating hormone [Hill, 1934; Chance *et al.* 1939], but the relevance of this to its apparent high galactopoietic activity is not immediately apparent, though there is just a possibility that this action is mediated by the anterior pituitary through the stimulating action of oestrogen produced by the ovary in response to the exogenous gonadotrophin. Pig, sheep and ox pituitary glands are all low in this type of gonadotrophic activity [Chance *et al.* 1939].

In the experiments entailing repeated administration of ox, sheep and pig pituitary extracts (Table 2, and Fig. 1) any slight initial galactopoietic activity of the sheep and pig extracts must have been quickly masked by the rapid decline in milk production which began about a week after treatment had begun. The possibility is clear that this fall was the result of rapid antihormone formation in the cows treated with heterologous material. We have never observed such a phenomenon in experiments involving the prolonged treatment of lactating cows with homologous glandular extracts. Antisera to heterologous preparations of prolactin have been prepared in rabbits and other animals [Young, 1938; Kabac, 1938; Bischoff & Lyons, 1939], and there is evidence that the administration to lactating mice of rabbit antiserum raised against ox prolactin induced depression of milk production [Young, 1938]. It is possible that antibodies to prolactin or to other pituitary factors concerned in the galactopoietic action of anterior-pituitary extracts appeared in our cows treated with sheep and pig pituitary extracts within 1-2 weeks of treatment, producing a state which was not only refractory to any galactopoietic action of the extract, but also inhibitory to the action of the relevant secretions of the animal's own pituitary gland [cf. Thompson, 1941]. Unfortunately, owing to the conditions under which these experiments were carried out, we were not able to obtain samples of blood from the animals for the purpose of testing for antihormone formation, so that no definite conclusion can be drawn on this question. It is clear, however, that whatever be the cause, any galactopoietic action of sheep and pig pituitary extracts was completely masked by the depressing action on milk production of prolonged treatment with these preparations. Accordingly, sheep and pig pituitary extracts are useless from the point of view of stimulation of milk production on a wide scale.

The action of the ox pituitary extracts in increasing milk production was much less in the present experiments than was the case with our earlier ones [Folley & Young, 1945*a*]. Possible causes of such a difference will not be discussed here, but are considered elsewhere in relation to the results of field trials [Folley & Young, 1945*b*].

#### SUMMARY

1. When single injections of pituitary extract were made into cows in declining lactation the results showed that horse pituitary gland was more active in stimulating milk production than was ox pituitary tissue. Sheep and pig pituitary extracts showed little or no activity under these conditions.

2. These results with respect to milk production cannot be correlated with the relative prolactin contents of the pituitary glands of the different species, which fact supports the belief that prolactin is not the only factor, nor necessarily the most important factor, concerned in the galactopoietic action of anterior-pituitary extracts.

3. When groups of cows were treated, at 2-day intervals for a period of 2½ weeks, with sheep and pig anterior-pituitary extracts a substantial and sustained depression of milk production occurred. The possibility is considered that this fall was the result of rapid antihormone formation to heterologous glandular material.

We wish to express our thanks to Dr S. B. Bradshaw of Armour Laboratories for generous supplies of ox, sheep, and pig pituitary tissue. We are greatly indebted for the co-operation of Viscount Astor, on whose herd these experiments were carried

out, and to his agent Mr H. J. F. Smith, for willing assistance in these experiments. This work was carried out during the tenure by F. H. M. of a Research Grant from the Agricultural Research Council and the expenses were defrayed by special grants from the Agricultural Research Council and the Medical Research Council, to whom our thanks are due.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 1. INTRODUCTION

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(Received 13 October 1944)

The first experiments showing that the feeding of dried thyroid gland to cows caused an increase in milk secretion were made in Canada in 1931 and 1932 by Dr W. R. Graham, under Prof. H. D. Kay's direction [Graham, 1934a]. The investigation was continued at the National Institute for Research in Dairying in 1934 and 1935, when it was shown that subcutaneous injections of thyroxine were also active [Graham, 1934b; Folley & White, 1936; some chemical work by Jones, 1935] in producing large increases in volume and also in fat percentage of the milk of cows past the peak of lactation. Since then these findings have been confirmed and extended in other laboratories. Unfortunately neither the feeding of dried thyroid gland nor the parenteral administration of thyroxine was a method which could be generally used on the commercial farm for stimulation of milk secretion.

In 1939 Ludwig & von Mutzenbecher showed that casein, iodinated under specific conditions, on alkaline hydrolysis yields thyroxine. Correlation of these facts led to the suggestion by Prof. F. G. Young, embodied in a report to the Endocrinological Committee of the Agricultural Research Council by Prof. Young and Dr S. J. Folley in September 1940, that the administration by mouth of iodinated proteins to increase the milk production of cows should be investigated. Preliminary experiments carried out by Dr Folley and Dr Bottomley gave encouraging results, and at this stage Prof. Kay and Dr Folley consulted Dr C. R. Harington, who undertook to look into the question of the provision of larger supplies of the iodinated material. In 1941, a conference called by the Agricultural Research Council reviewed the position, and a group under the chairmanship of Dr C. R. Harington arranged for chemical work to be undertaken in collaboration with Messrs Boots Pure Drug Co. Ltd., for assays on small animals to be made by Dr S. J. Folley and for field experimentation with cows to be carried out on a fairly large scale. Dr S. Bartlett, Mr T. Dalling, Dr S. J. Folley and Mr K. L. Blaxter collaborated in the field work.

Iodinated ox plasma was first used, but the field results were not satisfactory and work was started on the iodination of Ardein, the registered trade mark for the ground-nut protein produced by Messrs I.C.I. (Explosives) Ltd., and largely composed of the globulin arachin. A further field experiment with iodinated Ardein gave encouraging results, and large batches of the material were therefore prepared. Unfortunately, these were not satisfactory, and it was apparent that further extensive chemical work was necessary if Ardein were to be used.

In 1942 it appeared that casein would be available in bulk, and a third group of experiments on iodinated casein was begun. At this stage Dr A. S. Parkes took

over the organization of further supplies of material and arranged for more extended biological assays on small animals of the iodinated proteins. In this direction work on the induction of growth in thyroidectomized rats was undertaken by Dr I. W. Rowlands and on premature metamorphosis in tadpoles by Dr R. Deanesly, both at the National Institute for Medical Research, whilst work on the basal metabolic rate of guinea-pigs was continued by Dr S. J. Folley and Miss Emmett at the National Institute for Research in Dairying.

The results of this co-operative research are described in the papers which follow.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 2. PREPARATION AND PROPERTIES OF PHYSIOLOGICALLY ACTIVE IODINATED PROTEINS

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(Received 13 October 1944)

The first iodinated proteins which contained iodine in true chemical linkage were prepared by Blum & Vaubel [1898] and by Hofmeister in the same year; Hofmeister iodinated an aqueous solution of crystalline egg albumin and found that after repeated purification by dissolving in alkali and precipitating with acid, a product was obtained which contained nearly 9% iodine which could not be removed by physical means. Hofmeister suggested that tyrosine was involved in the reaction and showed that the protein no longer gave a Millon reaction. Acid hydrolysis of the protein gave an iodine-rich fraction which was insoluble in acid, soluble in alkali, and according to the author resembled in many ways Baumann's iodo-thyrin from thyroid. In 1899 Kurajeff prepared iodinated egg and serum albumins with iodine contents of about 12%, and he also observed that the Millon reaction was absent after iodination. In 1910 Oswald isolated di-iodotyrosine from hydrolysates of iodinated albumin and casein, thus revealing the principal reaction to which the binding of iodine by proteins is due; a second possible reaction of this kind is suggested by the observation of Pauly [1910] who showed that iodine can also be introduced into the iminazole ring of histidine.

Since further advances in this field depended largely upon the work done in the elucidation of the nature of the iodine compounds of the thyroid, this will be described briefly here. In 1915 Kendall showed that the products of alkaline hydrolysis of the thyroid could be separated into acid-soluble and acid-insoluble iodine-containing fractions; the acid-insoluble fraction was effective in the treatment of myxoedema and raised the normal B.M.R., whereas the acid-soluble fraction was inactive. In 1919 Kendall isolated a pure crystalline amino-acid, by hydrolysis of the thyroid with sodium hydroxide, which he named thyroxine. Harington [1926] isolated thyroxine in a greatly increased yield by baryta hydrolysis and showed that this iodine compound, which contains about one-third of the total iodine in the thyroid, can be quantitatively separated from the other iodine fraction by virtue of its great insolubility in dilute mineral acids; in the following year Harington [Harington & Barger, 1927] completed his proof of the constitution of thyroxine by synthesis; clinical tests using synthetic thyroxine showed that it was highly active in relieving the symptoms of myxoedema, thereby demonstrating that it is the thyroid hormone or its active principle. The acid-soluble iodine compound of the thyroid was shown by Harington & Randall [1929] to be di-iodotyrosine, and careful investigation of both fractions failed to bring to light any other iodine-containing compound.

The similarity between the physiological action of iodinated proteins and thyroid substance was first indicated by Wormser [1897] in experiments on the treatment of myxoedematous patients and thyroidectomized dogs with iodinated casein. This similarity was also demonstrated by Morse [1914], who found that iodinated proteins, like thyroid substance, accelerate the metamorphosis of tadpoles.

It had already been shown by Strouse & Voegtlin [1909] that di-iodotyrosine was ineffective in relieving symptoms of human myxoedema and cretinism; Rogoff & Marine [1916] found that acid-soluble and acid-insoluble fractions of thyroid hydrolysate described by Kendall [1915] behaved differently in their actions on tadpole metamorphosis, the former being inactive and the latter having an accelerating action. It might be supposed from these experiments that the relief of myxoedema and raising of the B.M.R. of mammals on the one hand, and the acceleration of tadpole metamorphosis on the other, are responses to an identical stimulus. It is demonstrable that this is only partially true, since it has been repeatedly shown [Morse, 1914; Abderhalden & Schiffman, 1923; Abelin, 1931; Hoffmann & Guderhatch, 1933] that di-iodotyrosine is able to accelerate tadpole metamorphosis. This effect has been studied quantitatively by Romeis [1922], Gaddum [1927], and Deanesly & Parkes [1945*a*]; the activity of di-iodotyrosine was found by Romeis to be 1/500th to 1/1000th, by Gaddum to be less than 1/1000th, and by Deanesly & Parkes to be 1/100th to 1/200th of the activity of thyroxine. Thompson, Alper, Thompson & Dickie [1934] showed that intravenous administration of di-iodotyrosine to myxoedematous patients over a period of 2-3 weeks in doses several hundred times as great as a single effective dose of thyroxine had no effect on the B.M.R. It appears therefore that the response in the tadpole metamorphosis test is not specific to thyroxine, the only substance which is known to produce the same response in mammals as thyroid feeding.

In 1939 Lerman & Salter showed that iodinated serum was effective in the treatment of human myxoedema and thyroidectomized rabbits, and further that after alkaline hydrolysis of the iodinated serum the activity could be concentrated into the acid-insoluble fraction; later they showed that the hydrolysed protein could be separated into fractions exhibiting the properties of thyroxine and those of di-iodotyrosine. Previously, between 1933 and 1936, Abelin had shown that an iodine-containing fraction could be obtained by baryta hydrolysis of iodinated albumin, which raised the B.M.R. of rats [Abelin & Florin, 1933]. This active fraction, which Abelin named homothyroxine, contained 43.4% iodine, resembled thyroxine in its physiological action, and caused depigmentation in hen's feathers [1934*a, b*]. The substance also caused moulting in hens and lessened the fall of temperature in guinea-pigs following novocaine injections [1934*c*]; homothyroxine could be further purified to give a crystalline compound similar in appearance to thyroxine [1936] but was not identified with thyroxine, and it was not until 1939 that Ludwig & von Mutzenbecher isolated thyroxine from casein which had been iodinated under carefully controlled conditions. The iodination was carried out in bicarbonate buffer at 38°, and the amount of iodine added was approximately equivalent to 4 atoms per molecule of tyrosine in the casein. Crystalline thyroxine was isolated from the product of this reaction, and the authors showed that deviation from these conditions, such as addition of more or less iodine, gave products from which less or no

thyroxine could be isolated; the thyroxine was identified by its physical properties and by analysis. A peptic hydrolysis product of iodinated casein was tested on guinea-pigs, a similarly treated thyroid product being used as a control. The authors found that the casein hydrolysate had about one-twentieth of the activity of the thyroid product per mg. of iodine. From this work it may be concluded that as in the thyroid, the active principle of artificially iodinated proteins is thyroxine.

Muus, Coons & Salter [1941] found that the physiological activity of iodinated serum (measured by tests on myxoedematous patients) first appeared when 2 atoms of iodine per molecule of tyrosine had been added and rose to a maximum with the addition of 4 atoms of iodine; thereafter further additions of iodine had little effect on the activity of the product, though the results were not well defined. These authors also showed that under the conditions used, iodination occurs preferentially in the tyrosine molecule.

Reineke, Williamson & Turner [1942] showed that the activity of iodinated casein in accelerating the metamorphosis of tadpoles (*Rana pipiens*) increases with progressive iodination until 4-5 atoms of iodine per molecule of tyrosine have been added; further addition of iodine decreases the activity of the protein. This confirmed Ludwig & von Mutzenbecher's observation on the relationship between the yield of thyroxine and the amount of iodine added during iodination of casein. Further, Reineke, Williamson & Turner [1943] studied optimal conditions for iodinating casein and soy-bean protein; they confirmed their previous results and discovered that iodination of casein at a raised temperature (70°) or subsequent incubation of the treated protein at this temperature yielded a product with a markedly increased biological activity; they also claimed that the activity could be further increased by carrying out the reaction in presence of a brass stirrer [Reineke & Turner, 1942]; we have been unable to confirm this observation; the acid-insoluble iodine contents of brass-stirred preparations of iodinated casein (Table 8) do not differ significantly from other preparations in the series NCB1-62, and tadpole metamorphosis tests on six of these brass-stirred preparations show no significant difference from the other preparations [Deanesly & Parkes, 1945*b*].

It was suggested by Reineke & Turner [1942] that iodinated proteins may have a thyroid-like action on milk secretion of cows; this suggestion arose from the discovery by Graham [1934], confirmed by Folley & White [1936], that administration of dried thyroid or injection of thyroxine caused a considerable increase in the milk secretion of cows. In a comprehensive work on the preparation and properties of iodinated proteins these authors have determined the optimal conditions of iodination for production of biological activity; their products have been tested by their action on the metamorphosis of tadpoles, on oxygen consumption in guinea-pigs and on thyroidectomized goats. Short-term (3-day) feeding experiments on cows have shown that iodinated proteins increase the total milk yield and the percentage of fat. Thyroxine in a yield as high as 0.4% [Reineke & Turner, 1943] has been isolated by baryta hydrolysis of iodinated casein.

The work to be described in this paper was started in May 1941, and had the same theoretical basis as that of Reineke & Turner referred to above; it forms the chemical basis of the extensive experiments on cows reported in the accompanying papers by K. L. Blaxter [1945*a, b*].



## PLAN OF EXPERIMENTS

Speculation on the mode of formation of thyroxine in proteins plays an important part in the choice of a starting material for the preparation of a biologically active iodinated protein. In 1927 Harington & Barger suggested that the most likely biological precursor of thyroxine was di-iodotyrosine, from which it would follow that a protein with a high tyrosine content will probably favour di-iodotyrosine and consequent thyroxine formation, after iodination. Ludwig & von Mutzenbecher showed [1939] that thyroxine could be obtained from other iodinated proteins besides casein, such as plasma proteins and cdestin, although casein gave the best yields. That high tyrosine content is not the only factor involved in thyroxine formation was shown by the same workers who found that silk fibroin, with the highest tyrosine content of all their starting materials, gave little thyroxine after iodination and alkaline hydrolysis. Presumably other factors such as solubility at the optimum pH or molecular structure of the protein are concerned in the formation of thyroxine.

In considering the possible large-scale application of iodination of proteins to increase the milk yield of cows the first question to be decided was the protein to be used. When this work was begun, early in 1941, it was doubtful whether adequate supplies of casein would be available, and attention was therefore directed to other proteins; the first choice fell on ox plasma, for the two reasons that Ludwig & von Mutzenbecher had found it to be suitable for thyroxine formation and that it was available in quantity. Some laboratory experiments were accordingly carried out with ox plasma under slightly varying conditions of iodination in order to determine those which were most satisfactory.

In all iodination experiments to be described the tyrosine content of the protein was determined either by the method of Folin & Marcuzi [1929] or by that of Folin & Ciocalteu [1927]. Products were analysed by the method of Harington & Randall [1929], total iodine and acid-insoluble iodine being determined on all preparations; the values of the absolute amount of acid-insoluble iodine and of the ratio of acid-insoluble to total iodine were taken as the best available guide in deciding whether a preparation was suitable for biological tests on cows, although it is recognized that no quantitative relationship exists between physiological activity and acid-insoluble iodine of iodinated proteins. (Compare Abelin & Neftel [1938] and the experiments on the addition of large amounts of iodine described below.)

*Iodination of ox plasma*

In the laboratory experiments on quantities of about 1 l. of ox plasma the yields of iodinated protein were good, being of the order of 100 % calculated on the weight of protein in the starting material (see Table 1), and the acid-insoluble material found varied from 0.6 to 1.0 %. The activity of this series of preparations was tested by its action on the B.M.R. of guinea-pigs, and although the numbers of animals used were small, the results suggested that it would be worth while pursuing the work on a larger scale. It has been found recently by Deanesly & Parkes [1945b] that two of the laboratory preparations of iodinated ox plasma, L1(b) and L3(b), are active in accelerating the metamorphosis of tadpoles. Preparations from 30 to 40 gal. of ox plasma were therefore made, all of which, however, had a considerably

lower acid-insoluble iodine content than the laboratory-scale experiments (0.1–0.5 %). Moreover, cow-feeding experiments failed to show any effect on milk yields [Blaxter, 1945*a*]. Nevertheless, Deanesly & Parkes [1945*b*] have found that one of these preparations, iodinated plasma N2, with 0.7 % acid-insoluble iodine (see p. 229), is active in tadpoles.

This failure led to a search for another protein as the starting material, and it was suggested that Ardein (the trade name for a mixed protein from ground nut prepared by Messrs I.C.I. (Explosives) Ltd., Ardeer) might be suitable and was available in quantity.

#### *Iodination of Ardein*

Preliminary experiments were carried out on the iodination of Ardein, the conditions being varied in order to determine those which were most favourable. The first laboratory preparations (Table 2, Batches L1 to L6) were made from a sample of Ardein containing 6.4 % tyrosine, and products were obtained with higher acid-insoluble iodine contents than any obtained from subsequent batches of Ardein with lower tyrosine contents.

An attempt was made to reduce the acid-soluble iodine content of the product which contained a high proportion of iodide. A batch of 25 g. of Ardein was treated with 3.5 atoms of iodine per molecule of tyrosine; the solution was then halved. The first half (preparation iodinated Ardein L6(*a*)) was incubated for 16 hr. at 37°; more iodine up to 4.4 atoms was added to the second half (L6(*b*)) and the solution was incubated at 37° for 16 hr.; the two preparations were then worked up in the same way, and it was found on analysis that the portion to which 3.5 atoms of iodine had been added contained 14 % less acid-soluble iodine while the acid-insoluble iodine had not been materially altered. This was considered a satisfactory indication that good products would be obtained from Ardein by addition of 3.5 instead of 4.4 atoms of iodine.

In all laboratory experiments, recovery of the iodinated product was good (Table 2).

A large laboratory preparation of iodinated Ardein (LXm) was made, starting from 10.75 kg. of Ardein. The product contained somewhat less acid-insoluble iodine (0.66 %) than was obtained from small preparations, but was thought suitable for preliminary cow-feeding experiments. The preparation was found to be active on cows [Blaxter, 1945*a*, Exp. 2] and led to the attempted preparation of iodinated Ardein on a manufacturing scale; it was again found, as in the case of iodinated plasma proteins, that both the yield and the acid-insoluble iodine content of the product fell when the size of the experiment was greatly increased (Table 3). An active product (iodinated Ardein N4MB) was nevertheless obtained which, when administered orally to cows, produced a significant rise in the milk yield [Blaxter, 1945*a*, Exp. 4].

Further, an attempt was made to concentrate the active principle of iodinated Ardein by alkaline hydrolysis. A few small experiments led to a large preparation (Table 4, LXm concentrate) which contained 2.7 % acid-insoluble iodine; when fed to cows, however, the preparation was highly unpalatable and inactive [Blaxter, 1945*a*, Exp. 2] and the experiment was not pursued.

It will be seen from figures given in the experimental section that the acid-insoluble iodine is generally not much more and is sometimes less than one-sixth of the total

iodine; of the acid-soluble iodine a large proportion is present in the form of inorganic iodide which not only has no beneficial effect on the milk yield but is mainly responsible for the symptoms of iodism produced when these iodine-containing products are fed over even relatively short periods. Attempts were therefore made to remove the inorganic iodine from the treated protein by redissolving the moist cake obtained after the first acid precipitation in dilute alkali, and reprecipitating at the optimum pH; this was not satisfactory, since it appears that at the pH of maximum precipitation iodide is adsorbed by the protein. So far no workable method has been found to achieve this object on a large scale, although in laboratory experiments dialysis or precipitation of the protein with trichloroacetic acid gives a product which is virtually free from inorganic iodide (Table 5).

Simultaneously with the large-scale preparation of iodinated Ardein a number of laboratory experiments were carried out on this protein, controlled in some instances by identical experiments on casein to discover whether the yield of active material could be increased by varying the temperature or the proportion of iodine added per molecule of tyrosine; the temperature of iodination was raised to 50, 60 and 70° (Table 6), iodination being carried out in borate buffer since it was thought that at such high temperatures decomposition of bicarbonate to carbonate would raise the pH beyond the optimum required for the preparation of active products. The three products obtained contained less acid-insoluble iodine than those prepared at 38° in bicarbonate buffer; this is contrary to the observation of Reineke, Williamson & Turner [1942] on the beneficial effect of temperature of iodination on the preparation of iodinated proteins, assuming that the biological activity and acid-insoluble iodine content bear some relationship to each other. This is not a valid assumption beyond certain limits, as will be shown, but in the experiments described above the amount of iodine added was the same as in previous experiments, and the conclusion that a product with lower acid-insoluble iodine would have less biological activity was considered justifiable. It is possible that the presence of borate in the experimental solution exerts an inhibitory effect on the formation of thyroxine, and in this connexion it might be noted that in one other iodination of Ardein when bicarbonate buffer was used in conjunction with phosphate buffer, the product obtained had an acid-insoluble iodine content of only 0.36%.

The ratio of added iodine to the tyrosine content of the protein was varied (Table 7), and large increases in the acid-insoluble iodine contents of the products were obtained by adding 15–25 atoms of iodine per molecule of tyrosine; these increases, however, did not represent an increase in thyroxine, since the compounds, when tested on guinea-pigs, showed no biological activity whatsoever. A control experiment on casein to which 25 atoms of iodine per molecule of tyrosine were added yielded a product which contained 3.7% acid-insoluble iodine, but was also quite inactive in guinea-pigs; this preparation has since been shown by Deanesly & Parkes [1945*b*] to be inactive in promoting metamorphosis in tadpoles.

As a starting material for the preparation of a physiologically active iodinated protein, Ardein has several disadvantages; yields of the treated protein are not always good, the biological activity is not great even in the best samples so far obtained, the treated protein has quite a high degree of unpalatability for the cow and it appears to be impossible to obtain products with similar analytical and

physiological properties using apparently identical experimental conditions (Table 3). For example, batches N3MB and N4MB of iodinated Ardein, prepared under the same experimental conditions, had acid-insoluble iodine contents as far apart as 0.3 and 0.88 %; similarly, batches N5+6MB and N9+10MB differ between 0.1 and 0.31 % in acid-insoluble iodine. This may be partly due to uneven sampling of the iodinated proteins for analytical purposes. It will be shown that some products contain fractions with different acid-insoluble iodine contents, and with a mixture of proteins such as Ardein these differences may be particularly marked. It is therefore quite possible that the first fraction to be precipitated by acid may not be representative of the entire product. That this can be so is shown in the iodinated casein series; small samples of NC4 and NC5 were dried for analysis before the main bulk had been finally dried and ground. The acid-insoluble iodine values were 2.6 and 2.0 % respectively. The two preparations after drying were mixed and the acid-insoluble iodine content of the mixture was 1.6 %. Since the first analytical samples of NC4 and NC5 have been used by Deanesly & Parkes [1945*b*] for tadpole metamorphosis tests, these values are included in the experimental section as well as that of the pooled mixture.

For these reasons further attempts were made in 1942 to obtain casein in bulk since this protein is known to give a good yield of product on iodination which has a higher degree of activity than that of any iodinated protein so far studied. These efforts were successful and work on Ardein was temporarily abandoned in favour of casein, on which experiments were started early in 1943.

### *Iodination of casein*

Initial experiments were done on milk powder which was immediately available, but the products had a low acid-insoluble iodine content and yields were poor. One attempt was made to increase the acid-insoluble iodine content of the treated protein by incubating the product after iodination with hydrogen peroxide and horse-radish peroxidase, since it has been shown by Westerfeld & Lowe [1942] that cresol is oxidized (amongst other products) to a diphenyl ether and it was thought that the enzyme might increase the production of thyroxine from di-iodotyrosine. The experiment was unsuccessful and was not pursued.

Argentina lactic casein was used in all subsequent experiments and gave, on the whole, products with satisfactory acid-insoluble iodine contents in reasonably good yield. Iodinated casein NC4 has been shown [Blaxter, 1945*b*] to be active in increasing the milk secretion of cows, and most of the preparations have been shown by Deanesly & Parkes [1945*b*] to accelerate metamorphosis in tadpoles.

A further series of sixty-two batches of iodinated casein has been prepared (NCB 1 to NCB 62). The acid-insoluble iodine contents of the preparations are still variable on batches made under the same experimental conditions, but all batches have been shown to possess activity in promoting tadpole metamorphosis by Deanesly & Parkes [1945*b*], and a large part of this series of preparations has been used in a field experiment to be described elsewhere.

## EXPERIMENTAL

*Series 1. Iodinated ox plasma**Iodinated plasma (small scale)*

The total protein and tyrosine contents of the plasma were estimated on the first batch used; thereafter the protein content only was estimated and the tyrosine content assumed to be the same. The first preparation (L1(a), Table 1) was diluted to contain 2.5 % protein; subsequent batches were not diluted. Sodium bicarbonate was added to 0.75 %, the solution was warmed to 37° and iodine (4.4 atoms per molecule of tyrosine) was added either as a fine powder or as a 5 % solution in 10 % KI. The rate of addition of iodine varied with the size of the preparation and was controlled by the rate of disappearance of iodine. Von Mutzenbecher's condition of 4 hr. for the addition of iodine to 100 g. protein was found suitable. At the end of iodination some preparations were incubated at 37°. The solution generally contained small clots with adherent iodine; these were removed by filtration and the product was precipitated with HCl to about pH 4.5 and in some cases steam coagulated at 85°. An alternative method of isolation was precipitation with methylated spirit (containing 0.8 % acetic acid) at a final concentration of 50 % alcohol. The product was filtered on a Büchner funnel with gentle suction and dried in air or *in vacuo*.

Table 1

Batch no.	Weight of starting material g.	State of iodine	Incubation	Method of isolation	Yield g.	Total iodine %	Acid-insoluble iodine %
<i>Iodinated plasma</i>							
L1(a)	100	Powdered	None	HCl cold	100	5.75	0.9
L1(b)	10	"	3 days	Alcohol+acetic acid	8	5.3	1.5
L2	75	"	None	HCl+steam 85°	74	6.0	0.7
L3(a)	16.6	"	3 days	Alcohol+acetic acid	16	5.9	1.18
L3(b)	16.6	"	None	HCl+steam 85°	17	5.8	0.6
L4(a)	16.6	"	3 days	HCl+steam 85°	17	7.94	0.84
L4(b)	25	Solution	None	Alcohol+acetic acid	25	6.82	0.9

It appears from the above table that incubation after iodination favours the formation of acid-insoluble iodine compounds and that these are to some extent destroyed by isolating by steam coagulation.

The variability in the total iodine content of the preparations, to all of which 4.4 atoms of iodine per molecule of tyrosine were added, may be due in part to differences in the amount of iodide adsorbed on the protein, since none of the preparations was dialysed. This is not, however, the whole explanation, since in the iodinated casein series batches NC3, NC4 and NC5, to which the same amounts of iodine per molecule of tyrosine had been added, were analysed for total iodine before and after dialysis; after dialysis the total iodine was 7.3, 7.0 and 5.9 % respectively.

*Iodinated plasma N1 (large scale)*

35 gal. of oxalated ox blood, diluted with an equal volume of normal saline, were centrifuged in a Sharples centrifuge, 40 gal. of plasma containing 3.5 % protein (total 6.35 kg.) being obtained.

After the pH had been adjusted to 8.5 with 3.6 kg. of  $\text{NaHCO}_3$  and 300 ml. of 10N NaOH, the plasma was heated to 37° in a 100 gal., enamel-lined steam-pan and finely powdered iodine added with stirring, 160 g. during the first hour, 370 g. during the second, and 490 g. spread over the third and fourth hours. The total weight used, 1020 g., was 7½% more than the theoretical amount required for 4 atoms of iodine per molecule of tyrosine. The solution was stirred for a further 2 hr. when a lump of denatured protein mixed with solid iodine was removed, ground up to a paste and then returned to the solution. After a further 3 hr. stirring the iodinated protein was precipitated by the addition of 10% HCl to approximately pH 3 and 84 lb. of NaCl.

The precipitate was centrifuged off in a Delaval separator and purified by solution in 30 gal. of water with 650 ml. of 10N NaOH followed by reprecipitation with 10% HCl and 60 lb. of NaCl at pH 3. The precipitate was collected by centrifuging and dried at 37°. Weight, 8.0 kg. Analysis: total I, 3.0%; acid-insoluble I, 0.1%; and ash, 30%.

#### *Iodinated plasma N2*

38 gal. of undiluted, but slightly haemolysed plasma containing 14.85 kg. of protein were treated with powdered iodine at pH 8.5 and 37°. The iodine was added at a rate of 6 g. every ¼ hr. for 92½ hr. until 2220 g. had been added. The solid material which collected at the bottom of the pan was ground up and returned to the solution every 24 hr. Stirring and incubation were continued for 24 hr. more, when the iodinated protein was precipitated with 10% HCl and NaCl at pH 3. It was purified by solution in water at pH 8 and, after reprecipitation at pH 4 with 10% HCl, by coagulation at 85° for 10 min. The collected dried iodinated protein weighed 12.3 kg. Analysis: total I, 5.6%; acid-insoluble I, 0.7%.

#### *Iodinated plasma N3*

This was prepared from 74 gal. of plasma (protein, 26.2 kg.) as above. Yield, 30.9 kg. Analysis: total I, 3.48%; acid-insoluble I, 0.27%.

#### *Iodinated plasma N4*

26 gal. of plasma (protein, 9.6 kg.) were iodinated with 5% I in 10% KI solution, 100 ml. being added every 20 min. for 96 hr. Very little precipitation of denatured protein occurred. Purified as above, the yield was 12.0 kg. Analysis: total I, 5.4%; acid-insoluble I, 0.4%.

### *Series 2. Iodinated Ardein*

#### *Iodinated Ardein (small scale)*

Finely ground Ardein was suspended in 0.75% bicarbonate solution and dissolved by adding the minimum amount of NaOH; the alkali was then neutralized with HCl, the final concentration of the protein being 5%. The solution was stirred mechanically in a bath at 37–38° and iodine was added as a 5% solution in 10% KI except in the case of L1(a) when powdered iodine was used. The product was precipitated by addition of HCl to pH 4.0–4.5 and was purified by redissolving in dilute alkali and reprecipitating with aqueous alcohol and acetic acid only in preparations L1(a) and L4. The products were air-dried or dried *in vacuo*. Iodinated Ardein LXm was prepared in 3 kg. lots, each one being incubated at 38–42° for 20 hr.

Table 2

Batch no.	Weight of starting material g.	Tyrosine %	Atoms I per mol. tyrosine	Incubation time	Yield g.	Total iodine %	Acid-insoluble iodine %
Iodinated Ardein							
L1 (a)	100	6.4	4.4	None	90	6.95	1.15
L3	25	6.4	4.4	5 days	Not recorded	8.58	1.27
L4	20	6.4	4.4	16 hr.	20	6.68	0.97
L5	25	6.4	4.4	19 days	20	9.92	1.37
L6 (b)	12.5	6.4	4.4	16 hr.	12	6.94	1.26
L6 (a)	12.5	6.4	3.5	16 hr.	12	6.25	1.35
L9	25	4.55	3.5	None	24	6.26	0.93
LXm	10.75 kg.	4.50	3.5	20 hr. per 3 kg. lots	10.63 kg.	6.22	0.66

A batch of iodinated casein CLXm was prepared under the same conditions as iodinated Ardein LXm; 2.75 kg. casein gave only 1.03 kg. of product, with total I 11.1% and acid-insoluble I 2.0%. This product was practically inactive in cow-feeding experiments [Blaxter, 1945*a*, Exp. 2], but the validity of this biological test is doubtful since an outbreak of mastitis occurred in the cows used in the experiment.

#### *Iodinated Ardein (large scale)*

A typical batch was processed as follows:

40 lb. of Ardein were mixed in the dry state with 12 lb. of  $\text{NaHCO}_3$  and water slowly added with good stirring to 80 gal. The solution was then heated to  $39 \pm 2^\circ$  in a stirred, enamel-lined steam-pan and treated with  $5\frac{1}{2}$  gal. of a solution of 10% I in 12% KI. This was added continuously at the following rates:

Hr.	0-2	2-24	24-48	48-72	72-96	96-106
ml./hr.	1200	350	320	200	100	50

After the addition of the iodine, the mixture was heated for a further period up to 24 hr. In some cases a slight ammoniacal smell developed and the incubation

Table 3

Batch no.	Weight of Ardein lb.	Incubation time hr.	Purification	Main bulk yield lb.	Total iodine %	Acid-insoluble iodine %	Salt fraction yield lb.	Total iodine %	Acid-insoluble iodine %
Iodinated Ardein									
N1 MB	40	24	0.1% acetic acid	24 $\frac{1}{2}$	6.3	0.7	3 $\frac{1}{2}$	—	—
N2 MB	40	24	1.0% acetic acid	30	7.63	0.3	1	—	—
N3 MB	40	24	Reprecipitation	24	5.8	0.3	9 $\frac{1}{2}$	5.40	0.50
N4 MB	45	24	"	27 $\frac{1}{2}$	5.76	0.88	3 $\frac{1}{2}$	3.61	0.50
N5 MB	45	12	1.0% acetic acid	49	4.85	0.1	3	3.58	0.27
N6 MB	45	12							
N7 MB	45	24	"	53	4.18	0.22	1 $\frac{1}{2}$	—	—
N8 MB	45	24							
N9 MB	26 $\frac{1}{2}$	12	"	34 $\frac{1}{2}$	5.51	0.31	1 $\frac{1}{2}$	—	—
N10 MB	26 $\frac{1}{2}$	12							
N11 MB	40	6	"	24	5.85	0.29	1	—	—
N12 MB	10	6	Reprecipitation	7 $\frac{1}{2}$	3.91	0.085	—	—	—
N13 MB	10	16	"	5 $\frac{1}{2}$	3.78	0.074	—	—	—
N14 MB	20	16	"	13 $\frac{1}{2}$	6.21	0.19	—	—	—

was immediately stopped. The solution was cooled to 20°, a few ml. of capryl alcohol added and the product precipitated by addition of 5*N* HCl to pH 3.5; samples of the mother liquor were tested for complete precipitation by the addition of NaCl. The precipitate was collected in a rubber-lined basket centrifuge and purified by one of the following methods: (a) by suspension in 0.1 % acetic acid, (b) in 1.0 % acetic acid, or (c) by solution in water at pH 8-9, using 10*N* NaOH, followed by reprecipitation with 5*N* HCl at pH 3.5. The product was again collected by centrifuging and dried in a vacuum oven at 50°.

A further small quantity, 'salt fraction' (SF), was obtained by adding NaCl (10 %) to the purification mother liquors.

*Attempt to concentrate the active fraction of iodinated Ardein by alkaline hydrolysis*

Different batches of iodinated Ardein were hydrolysed by heating at 95-100° for 1 hr. in NaOH solutions of different concentrations; the solutions were then cooled and acidified to pH 4.5 (approx.) and the precipitates were collected, washed and dried in air or *in vacuo*.

Table 4

Starting material			Hydrolysis	Product			
Batch no.	Weight g.	Acid-insoluble iodine %		Batch no.	Yield g.	Total iodine %	Acid-insoluble iodine %
Iodinated Ardein							
L3	8	1.27	0.5 <i>N</i> NaOH	Concentrate 1	0.62	10.1	3.1
L3	8	1.27	0.25 <i>N</i> NaOH	Concentrate 2	1.32	7.34	1.43
L1(a)	5	1.15	1.0 <i>N</i> NaOH	Concentrate 3	1.33	13.32	4.6
L1(a)	5	1.15	2.0 <i>N</i> NaOH	Concentrate 4	1.15	12.7	4.96
LXm	8.4 kg.	0.66	0.9 <i>N</i> NaOH 3.2 kg./2 hr.	LXm concentrate	772	5.6	2.7

*Attempt to remove inorganic iodine from iodinated Ardein*

Iodinated Ardein preparations were (a) suspended in water and dialysed against running tap water for 16 hr., or (b) redissolved in water with dilute alkali and precipitated with trichloroacetic acid to a final concentration of 5 %. Recovery of about 90 % was obtained in all cases.

Table 5

Starting material	Total iodine %	Treatment	Total iodine %
Iodinated Ardein N1 MB	6.3	Dialysis	2.58
Iodinated Ardein N1 MB	6.3	"	1.72
Iodinated Ardein N1 MB	6.3	Trichloroacetic acid	2.15
Iodinated Ardein N2 MB	7.3	"	2.10

*Effect of temperature of iodination on the acid-insoluble iodine content of iodinated Ardein*

Ardein was iodinated (a) in borate buffer at pH 8.5 and (b) in bicarbonate solution at raised temperatures. In bicarbonate solution the pH rose from an initial value of 8.2 to a final value of 9.3.



Table 6

Batch no.	Weight of starting material g.	Buffer	Temperature	Atoms I per mol. tyrosine	Yield g.	Total iodine %	Acid-insoluble iodine %
Iodinated Ardein							
LF50	2	Borate	50°	3.5	1.7	3.6	0.35
LF60	2	"	60°	3.5	1.6	3.76	0.38
LF70	2	"	70°	3.5	Not recorded	3.97	0.41
N 14 MB	20 lb.	Bicarbonate	70°	4.3	13½ lb.	6.24	0.19

*Effect of excess of iodine on the acid-insoluble iodine content of iodinated proteins*

Ardein was iodinated in bicarbonate buffer at pH about 8.3 at 37° with large amounts of added iodine in KI solution. After about 5 atoms of I had been added it was found necessary to add small amounts of bicarbonate both to maintain the pH and to make the iodine react. The protein solutions were worked up as usual, but sodium metabisulphite was added to the alkaline solution before acidification if there was any unreacted iodine in solution. 2-5 g. lots of Ardein were used in these experiments and there was about 90 % recovery; casein was used in one experiment.

Table 7

Batch no.	Added I per mol. tyrosine	Total iodine %	Acid-insoluble iodine %
Iodinated Ardein LX	5	5.50	0.15
Iodinated Ardein LY	10	9.14	1.28
Iodinated Ardein LK	10	7.13	1.60
Iodinated Ardein LGEC	14	8.87	2.23
Iodinated casein LGEC	14	10.85	3.37
Iodinated Ardein LJ	15	9.95	2.20
Iodinated Ardein LPL	15	10.35	2.50
Iodinated Ardein LZ	25	9.20	2.56

*Series 3. Iodinated casein*

*Preliminary experiments*

(a) 100 g. of milk powder and 8.75 g. of  $\text{NaHCO}_3$  were dissolved in 700 ml. of water and heated to 38°. The solution (pH 7.6) was treated with 65 ml. of 10 % I in 12 % KI added slowly over 5 hr. and then incubated for 18 hr. at 38°. An equal volume of water was added and the iodinated casein precipitated at pH 4.2 with acetic acid and purified by reprecipitation. A portion equivalent to 21.6 g. of the original milk powder gave 6.2 g. of iodinated casein after drying. Analysis: total I, 5.93 %; acid-insoluble I, 0.29 %.

(b) 150 g. of milk powder, iodinated and incubated at 70° and purified as above, gave 52 g. of iodinated casein. The pH rose from 7.0 to 8.4 during the iodination, falling to 8.1 after incubation. Analysis: total I, 5.08 %; acid-insoluble I, 0.50 %.

(c) 50 g. of casein (Argentina lactie, 4.93 % tyrosine) were iodinated, etc., at 70° as above. Yield, 39.7 g. Analysis: total I, 6.77 %; acid-insoluble I, 0.71 %.

*Peroxidase experiment*

150 g. of milk powder were iodinated rapidly at 70° at pH 8.2, *N* NaOH being added continuously to keep the pH constant. The purified iodinated casein weighed 48 g. Analysis: total I, 5.93 %; acid-insoluble I, 0.39 %.

One-half of the iodinated casein (24 g.) was then hydrolysed on a steam-bath with 500 ml. of  $N$  NaOH for 36 hr. and, when cold, the remainder of the iodinated casein was added. The  $pH$  of the solution was then adjusted to 6.6, the solution warmed to  $38-40^\circ$  and 0.5 g. of a horse-radish preparation of peroxidase added. 6.5 ml. of  $H_2O_2$  (20 vol.) were added slowly over 2 hr. until a positive test with starch-iodide paper was obtained. After a further 2 hr. incubation the solution was poured into 3 l. of water and the iodinated casein precipitated at  $pH$  4.0. Yield, 26 g. Analysis: total I, 6.94%; acid-insoluble I, 0.47%.

#### Large-scale preparations—Batch NC1

40 lb. of Argentina lactic casein and 12 lb. of  $NaHCO_3$  were made into a paste with water and diluted to 80 gal. ( $pH$  7.45). After heating to  $39 \pm 2^\circ$  in a stirred, enamel-lined steam-pan, a solution of 5.6 lb. of I and 6.7 lb. of KI in 5 gal. of water was added at the following rates:

Hr.	0-2	2-24	24-48	48-72	72-93
ml./hr.	1200	350	350	250	150
$pH$	7.45	8.5	8.9	8.7	8.5

The solution was then incubated for 15 hr., cooled and the iodinated casein precipitated with 10% HCl at  $pH$  3.8. The precipitate was collected in a rubber-lined basket centrifuge, purified by solution in 75 gal. of water at  $pH$  8.5, using  $10N$  NaOH, and reprecipitation with 10% HCl from 1% NaCl at  $pH$  4.0, and dried at  $50^\circ$ . Yield,  $27\frac{1}{2}$  lb. Analysis: total I, 6.3%; acid-insoluble I, 1.0%.

#### Batch NC2

40 lb. of casein were dissolved in 80 gal. of water at  $pH$  8.0 by the addition of 1.1 l. of  $10N$  NaOH. After heating to  $40 \pm 2^\circ$ , 10% of I in 12% KI and 0.96  $N$  NaOH were added alternatively at the following rates:

Hr.	0-3	3-6	6-12	12-24	24-36	36-39
I, ml./ $\frac{1}{2}$ hr.	1000	750	500	350	250	200
NaOH, ml./ $\frac{1}{2}$ hr.	333	250	208	167	146	125

The solution was then incubated at  $65-70^\circ$  for 24 hr., 500 ml. (= 25 g. of casein) samples being taken at different times. The main yield, after purification, was 29 lb. Analysis: total I, 7.3%; acid-insoluble I, 1.2%.

The various samples when worked up gave:

Time, hr.	0	3	6	12	24	48
Yield, g.	18.3	15.7	15.0	15.1	15.9	14.5
Total I %	4.82	9.23	6.69	10.33	7.03	4.99
Acid-insoluble I %	0.51	0.53	0.56	0.53	0.59	0.76

The mother liquors from the first precipitation of the bulk were treated with NaCl to 10%. The precipitate was collected, dissolved in water at  $pH$  9.0 with  $10N$  NaOH and reprecipitated from 10% NaCl with 10% HCl at  $pH$  4.0. Yield, 205 g. Analysis: total I, 2.25%; acid-insoluble I, 0.03%.

The NaCl concentration of the above mother liquors was increased to 25% and gave 18.0 g. of a precipitate containing 1.97% I but no acid-insoluble I.

*Batch NC3*

Prepared from 40 lb. casein by iodination in  $\text{NaHCO}_3$  solution at  $39^\circ \pm 2^\circ$  as NCl. The incubation, however, was carried out at  $65-70^\circ$  for 24 hr. Yield,  $34\frac{1}{2}$  lb. Analysis: total I, 9.0%; acid-insoluble I, 2.7%. After dialysis of a solution in cellophane at pH 8 against tap water, the figures were total I 7.3%, and acid-insoluble I 2.7%.

*Batch NC4*

As NC3. Yield, 30 lb. Analysis: total I, 8.8%; acid-insoluble I, 2.6%. After dialysis: total I, 7.0%; acid-insoluble I, 2.5%.

*Batch NC5*

The casein was dissolved by addition of 10*N* NaOH to pH 8.0 before adding the  $\text{NaHCO}_3$  and iodinating, etc., as NC3. Yield, 31 lb. Analysis: total I, 8.25%; acid-insoluble I, 2.0%. After dialysis: total I, 5.94%.

*Iodinated casein NC4+5 (batches NC4 and NC5 pooled)* had total I 8.1% and acid-insoluble I 1.6%.

Table 8

Preparation no.	Total iodine %	Acid-insoluble iodine %	Preparation no.	Total iodine %	Acid-insoluble iodine %
NCB 1	7.35	1.57	NCB 32	8.42	1.52
2	8.11	1.70	33	8.50	1.59
3	9.08	1.71	34*	8.84	1.55
4	8.93	2.18	35	8.53	2.67
5	7.94	1.28	36	8.62	2.88
6	8.66	1.77	37*	8.79	2.29
7	8.38	1.11	38	8.11	2.48
8	8.47	1.26	39	8.19	1.76
9	6.77	0.90	40*	7.80	1.48
10	7.66	0.85	41	7.55	1.89
11	8.04	1.06	42	7.97	2.45
12	9.31	1.46	43*	8.65	2.14
13	8.49	1.86	44	8.06	1.89
14	9.99	1.78	45	8.31	2.16
15	8.29	1.74	46*	8.21	2.55
16	9.35	1.79	47	8.40	2.16
17	8.44	1.81	48	8.38	2.38
18	8.12	1.64	49*	8.31	2.79
19*	8.16	1.47	50	8.19	2.51
20	8.24	2.61	51	7.89	2.41
21	8.07	1.57	52*	8.14	2.70
22*	8.29	1.83	53	8.23	1.92
23	8.20	1.52	54	7.98	2.10
24	8.63	1.57	55*	8.07	2.27
25*	8.03	2.02	56	8.11	2.00
26	8.19	1.63	57	7.85	1.63
27	8.16	1.79	58*	7.60	1.30
28*	7.99	1.68	59	8.23	1.85
29	8.16	1.40	60	8.23	2.04
30	8.54	1.49	61*	8.27	1.93
31*	8.20	1.62	62	8.07	2.59

\* Iodination in presence of a brass stirrer [cf. Reinoko & Turner, 1942].

*Batch NC6*

The casein solution was made up as in NC5, but the iodination was carried out by the addition of small amounts of powdered I at the following rates:

Hr.	0-3	3-6	6-12	12-24	24-36
I, g./hr.	200	150	100	70	50

The preparation was then completed in the usual manner. Yield, 30 lb. Analysis: total I, 7.28 %; acid-insoluble I, 1.55 %.

*Batch NC7*

As NC6. Yield, 35 lb. Analysis: total I, 7.02 %; acid-insoluble I, 1.18 %.

*Iodinated casein batches* NCB1-NCB62 were prepared from 40 lb. of casein. 4.5 atoms of I per molecule of tyrosine were added in all cases and all preparations were incubated at 70°. Batches 2, 4, 6, 8, 10, 12, 14 and 16 were iodinated with 10 % I in 12 % KI solution. Powdered I was used in all other preparations. The total and acid-insoluble I values are given in Table 8.

## SUMMARY

1. The preparation is described of iodinated ox plasma, iodinated Ardein (the mixed protein from ground nut prepared by I.C.I. (Explosives) Ltd., Ardeer) and iodinated casein.

2. The total iodine and acid-insoluble iodine contents of all preparations have been determined; the acid-insoluble iodine value has been used as a guide in deciding whether any preparation should be used for biological experiments.

3. Iodinated plasma preparations contained up to 1.5 % acid-insoluble iodine, iodinated Ardein preparations contained up to 1.4 % acid-insoluble iodine, and iodinated casein preparations contained up to 2.8 % acid-insoluble iodine.

4. Iodinated products of all three proteins have been shown elsewhere to be active in increasing the milk secretion of cows and in accelerating tadpole metamorphosis.

5. Of the three types of iodinated protein obtained, iodinated casein was the most satisfactory to prepare on a large scale; better yields of product were obtained, and these showed the greatest biological activity.

6. No advantageous departure from the conditions given by Ludwig & von Mutzenbecher [1939] for the preparation of biologically active iodinated proteins has been found; the process of high temperature incubation of the iodinated product which was found by Reincke, Williamson & Turner [1942] to give highly active products has been used in the later preparations of iodinated casein.

We wish to express our gratitude to Dr C. R. Harington for his constant help and advice, without which this work would not have been possible. We also wish to thank Messrs I.C.I. (Explosives), Ltd., Ardeer, and Dr David Traill in particular, for gifts of Ardein.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 3. THE EFFECT OF IODINATED PROTEIN FEEDING ON THE LACTATING COW

### (i) THE EFFECTS OF PREPARATIONS OF LOW ACTIVITY AND OF IODINATED ARDEIN

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#### INTRODUCTION

That the thyroid gland is intimately concerned with the maintenance of milk secretion has been shown both by experiments involving the surgical removal of the gland, and by those in which the thyroid hormone has been administered to pregnant or lactating animals. Thyroidectomy in the lactating goat [Grimmer, 1918; Trautmann, 1919; Grimmer & Paul, 1930; Grueter, 1930] generally leads to a

depression, though not a cessation of milk secretion, while in the lactating cow, thyroidectomy has been shown to reduce the lactation period and milk yield [Spielman, Petersen & Fitch, 1944]. Thyroid extirpation experiments, are, however, often complicated by the presence of accessory thyroid tissue, and opinion is still divided on the lactational effect of thyroidectomy in the rat [Nelson & Tobin, 1937; Folley, 1938; Nelson, 1939], so that the most convincing evidence of the role of the thyroid in lactation is to be found in those experiments in which the dried gland or the pure hormone has been administered to normal animals. Weichert & Boyd [1934] found that experimental hyperthyroidism in the pregnant rat resulted in an earlier mammary development and an earlier initiation of the secretory phase than was found in comparable control animals, but the first demonstration of the effect of experimental hyperthyroidism on an established milk secretion was by Graham [1934 *a, b*]. In experiments with cows in the declining stage of lactation Graham found that feeding desiccated thyroid gland or the injection of thyroxine caused a marked temporary increase in both milk and fat production. This galactopoietic effect has since been confirmed in the goat and cow [Jack & Bechdel, 1935; Folley & White, 1936; Hernan, Graham & Turner, 1938; Smith & Dastur, 1940]. Contrary results by de Fremery [1936] were thought to be due to overdosage with thyroxine [Folley, 1940], and the stimulation of the milk production of lactating ruminants by the administration of thyroid gland or thyroid extracts can be regarded as firmly established.

For these reasons it became of interest to study the effect on lactation of iodinated proteins possessing thyroid-like activity of the type prepared by Ludwig & von Mutzenbecher [1939]. Bottomley & Folley prepared iodinated casein according to Ludwig & von Mutzenbecher's original method, and while their early experiments were not successful, they later demonstrated that the normal decline in yield of a single dairy cow could be prevented by feeding their preparation although no large increase in production occurred [1940, 1941]. Reineke & Turner [1942 *a, b*] have shown that increased milk production occurs when iodinated casein is fed. Increases in milk yield have also been demonstrated by Van Landingham, Henderson & Weakley [1944].

Large-scale experiments were commenced in the summer of 1941 by the 'Iodinated Protein Group',\* working under the auspices of the Agricultural Research Council to determine whether iodinated protein stimulation of milk production was possible under normal conditions of farm management, and whether such therapy was practicable from the aspect of the normal health of the cow. This has entailed a number of experiments conducted under the supervision of the Iodinated Protein Group, and this paper is concerned with the experiments carried out from July 1941 to May 1943, using the preparations which are described in the preceding paper by Pitt Rivers & Randall [1945].

In the first series of three experiments, briefly discussed in Part 1 of this paper, several centres in Great Britain co-operated, including the Agricultural Research Council's Field Station at Compton; Messrs Dartington Hall, Ltd. at Totnes; the National Institute for Research in Dairying at Shinfield; the Rowett Research

\* See the introductory paper of this series by the chairman of the group, Sir Joseph Barcroft, F.R.S. [Barcroft, 1945].

Institute at Aberdeen; and the University of Reading Farm at Sonning. A large number of workers spent a considerable amount of time collecting the essential information, and the co-ordination of the experiment was carried out at Reading where all experimental records were collected, summarized and interpreted, using statistical tests to determine the validity of the conclusions which were drawn. The second series of two experiments which is included in Part 2 of this paper was carried out at the National Institute for Research in Dairying, using more highly active preparations.

#### PART 1. EXPERIMENTS WITH IODINATED PROTEINS OF LOW THYROIDAL ACTIVITY, POTASSIUM IODIDE, AND THYROXINE

In the first experiment, conducted in July 1941, ox-plasma protein was used as the substrate for the preparation of an iodinated protein. This was because a continuous supply of blood could be assured, whereas casein was likely to be in short supply. Over ninety cows, which were used as experimental animals, were allocated to five treatments, consisting of the daily injection of 2.5 mg. of Na-thyroxine, 11.2, 22.5 or 45.0 g. of iodinated blood fed by mouth, or a control ration including dried blood which had not been iodinated. Following an initial control period of 21 days in which all necessary preliminary observations were made, the five experimental rations were fed for a period of 23 days. While the injection of thyroxine increased the resting pulse rate by 4.8 beats per minute and milk production by 0.70 lb. per day, even the highest dose of iodinated blood failed to stimulate metabolism or milk production. A comparison of the activity of the iodinated blood with that of a sample of Bottomley & Folley's preparation of iodinated casein indicated that the probable cause of the absence of a response was due to the very low—almost negligible—potency of the iodinated blood preparation.

A second experiment was therefore carried out in October 1941 on a smaller scale, in which the dosage of iodinated ox plasma of a somewhat higher potency was increased to 200 g. per day. Three other iodinated protein preparations were used. Ardein, a commercial preparation of the earth-nut *Arachis hypogaea*, was iodinated and two preparations used [Pitt Rivers & Randall, 1945], and iodinated casein was also given. Two further groups of cows were included. One received supplements of the pure proteins and acted as a control, while the cows in the other group were injected daily with 10 mg. of freshly prepared Na-thyroxine. The treatment lasted 14 days. Iodinated blood and the higher dose of iodinated Ardein were decidedly unpalatable and despite every artifice nothing could be done to ensure the complete consumption of the daily dose without forcible administration. This was accomplished by mixing the iodinated proteins with water and giving them forcibly in a draught, in the same way that medicine is given. Despite such drastic methods of administration most encouraging results were obtained. The mean daily increases in milk production were: thyroxine injection 5.61 lb. (24.9 %), 120 g. of iodinated Ardein 4.27 lb. (19.7 %) and 200 g. of iodinated blood 3.47 lb. (15.1 %). All these responses were highly significant statistically. 80 g. of iodinated casein gave an insignificant response of +0.49 lb. per day, but this result was complicated by an outbreak of mastitis in the group of animals concerned. 45 g. of the concentrated iodinated Ardein preparation gave no response in milk production. The



intakes of iodine by the cows which received the iodinated Ardein and iodinated blood supplements were considerable and slight symptoms of iodism occurred at the end of the treatment period. These rapidly disappeared when treatment stopped.

As iodinated Ardein had given the most promising results in this experiment, in July 1942 a further experiment was carried out with forty dairy cows, using fresh preparations of iodinated Ardein, and an attempt was made to incorporate the iodinated protein in a cattle cube which would be palatable to dairy cows. Three iodinated Ardein treatments were given, 120 g. or 80 g. of preparation N1MB and N2MB (mixed)\* or 80 g. of preparation N3MB, which had been precipitated and washed in an endeavour to remove the large amounts of acid-soluble iodine which it contained. Two control groups were used. Both received a cattle cube identical in composition with that containing iodinated Ardein, but containing the same quantity of pure Ardein. This was the only supplement for one group, while the other received 6 g. of potassium iodide in addition. When treatment started the cubes containing iodinated Ardein were eaten quite readily, but after 2 days most of the cows refused the whole or part of their ration. Every endeavour was made, by the use of highly palatable supplements and by adding condiments, to induce the cows to eat the cubes but with little success, for only a few of them would consume the whole of their ration. The control cows, including those fed potassium iodide, did not refuse their food, and so it was fully evident that the unpalatability of the iodinated Ardein caused the refusal. One interesting fact was that this unpalatability was not apparent until the second day of treatment, which indicates that the initial taste of the cube may not have been the sole factor concerned. From the records of milk production of the cows which consumed the whole of their ration there was no evidence to show that the iodinated Ardein had substantially affected milk production or metabolism. Potassium iodide feeding did not result in any change in milk production and produced no change in the metabolism of the cows which could be determined by careful observation.

Both iodinated Ardein and potassium iodide caused severe symptoms of iodine poisoning. These symptoms commenced during the 4th to 10th days of treatment and continued until treatment was stopped. The first symptoms to be observed were salivation and a watery discharge from the nostrils, and in some cases conjunctivitis and a discharge from the eyes were seen. The nasal discharge gradually thickened with continued treatment, becoming more catarrhal and cream-coloured. Later this discharge was even more profuse and its colour was a brilliant yellow. The mucous membranes of the nostrils became very inflamed. A similar cycle of changes occurred in the eye discharge, the exudate at first being clear and later a bright yellow. Frothing at the mouth was apparent in some cows and in others a scaly skin, but the discharges were the most pronounced symptoms. Even though such severe symptoms of iodism occurred, the cows behaved normally in every other respect, ruminating, grazing at pasture without appearing upset in any way and maintaining a normal milk production. From the records of food refusals it was possible to calculate the ingestion of iodine by each animal in the experiment. The minimum daily intake which caused iodism was 3.7 g., and this was the lowest

\* The nomenclature of iodinated protein preparations is that given by Pitt Rivers & Randall [1945], and takes into account the numerous laboratory preparations they have made.

intake by any cow. Of the thirty-two cows which received iodine in various forms, five showed no symptoms of intolerance. These consumed an average of 4.5, 4.6, 5.0, 6.4 and 6.9 g. of iodine per day during the experimental period of 21 days, and the fact that they did not show symptoms of iodism indicates a considerable variability between the iodine tolerance of individuals.

The results of these three experiments are summarized in Table 1, and while it is evident that a considerable increase in milk production and a definite thyroxine-like effect could be obtained by forcibly administering iodinated Ardein and iodinated blood to dairy cows, when comparable large doses of iodinated Ardein were given in eube form no milk-yield response resulted. A consideration of the acid-insoluble iodine content of the preparations and dosages calculated as acid-insoluble ('thyroxine') iodine, tended to indicate that a daily ingestion of over 0.75 g. of acid-insoluble iodine was necessary to elicit a milk-yield response. In view of the complete absence of a response in the third experiment when 0.58 g. of acid-insoluble iodine was fed, the fact that different methods of administration had been used in the successful experiment, and that the experiments had been conducted under widely differing conditions of feeding, management, and season, such a simple conclusion was not adequately justified at this stage.

Table 1. *Summary of results of Exps. 1-3, 1941-2*

Exp.	Preparation	Iodine analysis %		Daily dosage		Daily milk-yield change	
		Total	Acid-insol. I 'thyroxine'	g.	Acid-insol. I	lb.	%
1	Iodinated ox plasma N2+3 (mixed)	4.18	0.41	45.0	0.18	Nil	
1	Iodinated ox plasma N2+3 (mixed)	4.18	0.41	22.5	0.09	Nil	
1	Iodinated ox plasma N2+3 (mixed)	4.18	0.41	11.2	0.05	Nil	
2	Iodinated Ardein LXm	6.22	0.66	120.0	0.79	+4.3	+19.7
2	Iodinated plasma N4	5.4	0.4	200.0	0.80	+3.5	+15.1
2	Iodinated casein LXm	11.1	2.0	80.0	1.60	+0.5	+2.3*
2	Iodinated Ardein concentrate LXm	5.60	2.70	25.0	0.68	Nil	
3	Iodinated Ardein N1MB+N2MB (mixed)	7.03	0.48	120.0	0.58	Nil	
3	Iodinated Ardein N1MB+N2MB (mixed)	7.03	0.48	80.0	0.38	Nil	
3	Iodinated Ardein N3MB	5.68	0.36	80.0	0.43	Nil	
1	2.5 mg. Na-thyroxine injected subcutaneously	—	—	—	—	+0.7	+3.6
2	10.0 mg. Na-thyroxine injected subcutaneously	—	—	—	—	+5.6	+24.9

\* Result inconclusive due to mastitis in experimental animals.

It was, however, obvious that unless large increases in the potencies of the materials could be made the practical use of iodinated proteins was impossible, for the materials prepared were all highly unpalatable and such large doses had to be given before responses occurred that iodism soon occurred in a very severe form.

Further chemical work was therefore carried out [see Pitt Rivers & Randall, 1945] and a fourth batch of iodinated Ardein was prepared, 'N4MB'. The experiments which have been carried out using this material are reported in full.

## PART 2. EXPERIMENTS WITH IODINATED ARDEIN N4MB

## THE FIRST ASSAY OF IODINATED ARDEIN N4MB (EXP. 4)

Iodinated Ardein N4MB was a highly satisfactory preparation chemically for it contained 4.88% of acid-soluble iodine and 0.88% of acid-insoluble ('thyroxine') iodine.\* The 'thyroxine' iodine content was considerably higher than that of any other iodinated Ardein preparations used in the previous experiments.

In the account of the first three experiments it will be observed that there had never been a response in milk production to an iodinated protein which had been fed by mouth, while large responses had been found when the materials had been given forcibly in a liquid suspension. Because of the possible reduction of the calorogenic and galactopoietic potency of iodinated protein by bacterial and infusorial action in the rumen of the cow, and following the registration of an American patent [U.S.A. Patent No. 3681/1941] which described methods of protecting dried thyroid glands from decomposition in the rumen, the iodinated Ardein was coated with solid stearic acid before it was fed. This procedure has since been shown to have no effect on the potency of iodinated proteins, and such coating is not necessary to ensure a thyroxine-like effect in the bovine.

*Plan of experiment*

The experiment was conducted at the National Institute for Research in Dairying, using twelve mature cows as experimental animals. Three of these were Guernseys and the remainder were Dairy Shorthorns. They were all in mid- to late-lactation when the experiment began. Three experimental treatments were used.

(1) *Control treatment*, consisting of normal winter rations with the addition of 50 g. of normal Ardein.

(2) *50 g. treatment*, consisting of the same winter ration as (1) above with the addition of 50 g. of iodinated Ardein N4MB.

(3) *10 g. treatment*, consisting of normal winter rations with the addition of 10 g. of iodinated Ardein N4MB and 40 g. of normal Ardein.

The allocation of the cows to treatment was carried out by dividing them into four 'blocks' of comparable animals as alike as possible with regard to breed, age, productivity, body size and stage of lactation. The three animals in each block were then allocated to one of the three treatments by random methods, thus ensuring that comparable animals composed each treatment group. For a cow weighing 1000 lb., the normal winter ration consisted of 12 lb. of meadow hay and 40 lb. of marrow-stem kale. The hay ration was varied according to the initial live weight of the cow to make allowance for the greater nutrient requirement of the larger animal. This part of the ration was calculated to provide nutrients for maintenance and the production of 2.5 lb. of milk per day. For milk produced over this amount a mixture of concentrated foods was made and 0.425 lb. was fed for every pound produced. These rations of concentrated food varied for each cow, and the amounts fed were adjusted weekly on the basis of each cow's production in the previous 2 weeks. All the foods were weighed each day and a record was taken of any refusal of food

\* I am indebted to Mrs Pitt Rivers and Mr Randall for this information.

which occurred. The cows had access to a bare pasture for a few hours each day for exercise purposes.

The experiment was divided into three periods. During the first or initial control period (11 Oct. 1942 to 1 Nov. 1942) all cows received the normal winter rations and all preliminary records were taken. The second or 21-day experimental period (1 Nov. 1942 to 22 Nov. 1942) was when the iodinated Ardein was given. During the last or final control period (22 Nov. 1942 to 13 Dec. 1942) the experimental feeding was stopped and the rations of the cows were the same as in the initial control period.

### Results

A large quantity of numerical data was collected during the experiment including that related to milk production and composition, certain physiological measurements and signs of abnormal metabolism. Where possible the results have been analysed statistically. The method of analysis for each of the variables studied is complicated owing to the use of both control cows and control periods. In the case of milk yields, for instance, rather than use the method of co-variance analysis suggested by Bartlett [1935] the increases or decreases in yield in the experimental period for individual cows have been calculated and an analysis of the total variance of these differences has been made into 'blocks', 'treatment', and 'error' components. The response of a group of cows to treatment in terms of milk production can therefore be expressed as

$$(E_2 - E_1) - (C_2 - C_1),$$

where  $E_1$  = milk yield of experimental animals in the initial period,

$E_2$  = milk yield of experimental animals in experimental period,

$C_1$  = milk yield of control animals in the initial control period,

and  $C_2$  = milk yield of control animals in the experimental period.

This represents the difference between the decline in yield of initially paired cows which is due to a treatment effect, and the error of such a response is the standard error of the difference between the two factors in brackets after removal of the 'blocks' component of the total variance.

The tables which follow show both the mean values of the factors studied for each of the three experimental groups, together with responses to treatment calculated in this way. Statistical significance is expressed in the form of the odds that differences between treatment means as large as the observed differences would have arisen by pure chance in a sample of cows from a similar population.

### *The palatability of iodinated Ardein N 4MB*

Two cows receiving the 50 g. dose (cows D and CS) refused their concentrate + iodinated Ardein mixture on occasions. In the case of cow D this was largely due to the lack of palatability of the mixture, but, by careful mixing of the ration and by the use of various condiments, she was induced to eat her ration completely. Cow CS was severely affected by a head paralysis and she refused to eat both hay and kale during the period in which she refused iodinated Ardein. The other cows receiving the 50 g. dose (cows P7 and F31) did not refuse their ration on any occasion neither did the control cows nor those which were fed the 10 g. dose. The palatability of this iodinated Ardein therefore can be said to be moderately satisfactory.

*The effect on milk yield*

Milk yields were recorded twice daily to the nearest 0.25 lb. throughout the whole experiment. The mean daily milk yields and the mean daily responses in milk yield are shown in Table 2 and in Fig. 1.

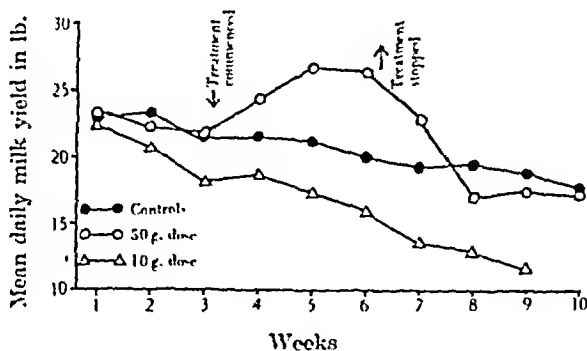


FIG. 1. The effect on milk yield of feeding iodinated Ardein N4MB for a period of 3 weeks.

Table 2. Mean milk yields in pounds per day

Period	...	Initial			Experimental			Final		
Week	...	1	2	3	1	2	3	1	2	3
Control		22.9	23.2	21.4	21.4	21.0	20.0	19.2	19.4	18.7
50 g. dose		23.2	22.2	21.7	24.3	26.6	26.3	22.8	16.5	17.3
10 g. dose		22.3	20.7	18.1	18.7	17.3	15.9	13.6	12.8	11.6
Response per day to 50 g. (lb.)					+3.2	+5.7	+6.6	+3.7	-2.8	-1.5
Response per day to 10 g. (lb.)					-0.5	-1.5	-2.0	-3.5	-4.5	—

It can be seen that feeding 50 g. of iodinated Ardein N4MB increased milk production very substantially. From daily plotting of the results the time/response curve indicated that the maximum occurred on the 21st day of treatment when the response was 6.98 lb. per day or 31.1%. Over the whole period the mean response per cow was 108.5 lb. of milk over the control cows, while inclusion of the first week of the final control period increases this to 134.3 lb. of milk. Statistical analysis showed that this increase was significant at odds of 550:1, and the increase was statistically significant in each of the three treatment weeks.

The cows fed the 10 g. dose gave a slight negative response which was not statistically significant. This was largely due to one cow (P19) whose yield fell rapidly during the experiment and which was dry at the end of the final control period. Fig. 1, however, indicates that there may have been a slight increase in production in the group, but the magnitude was too small to measure with any accuracy. During the final control period the yield of the cows which had received 50 g. of iodinated Ardein after remaining elevated for 5 days fell precipitously and reached a point slightly below the controls. In the last week of treatment a slight recovery was evident. This post-experimental depression of milk yield is similar to that observed by Folley & White [1936], and a similar depression of yield was noted in Exp. 2 above when the pure hormone was given subcutaneously. The 10 g. dose, however, also resulted in a slight depression of yield in the final period, again largely due to cow P19, but inspection of the yields of the remaining cows did not suggest

a thyroxine-like effect. It can be concluded, therefore, that a considerable increase in milk yield resulted when 50 g. of iodinated Ardein N4MB were fed, and in every way this increase resembled the increase found when the pure thyroid hormone or dried thyroid glands are given, but with a dose of 10 g. the scale of the experiment was not sufficiently large to detect a milk-yield response.

#### *The effect on milk composition*

In order to assess whether changes had occurred in either the fat content or the solids-not-fat content of the milk, samples of morning and evening milk were taken from individual cows on two consecutive days and the fat percentage and total solids percentage determined. Table 3 shows the mean fat percentage for the three treatment groups.

Table 3. *Mean fat percentage of the milk*

Period	...	Initial Mean of	Experimental			Final		
Week	...	3 weeks	1	2	3	1	2	3
Control		4.50	4.47	4.60	4.41	4.56	4.43	4.39
50 g. dose		4.35	4.16	4.69	4.63	5.01	4.55	4.45
10 g. dose		4.49	4.44	4.44	4.55	4.58	4.40	4.14
Response to 50 g. dose			-0.16	+0.24	+0.37	+0.61	+0.27	+0.21
Response to 10 g. dose			-0.02	-0.17	+0.15	+0.03	-0.03	-0.24

The fat percentage values were weighted according to milk yield in the usual way to determine the individual cow averages, and it was evident that there was an increase in the fat percentage during the treatment period in the cows which were fed 50 g. of iodinated Ardein per day. Statistical analysis of the responses to this dosage in the second and third experimental weeks shows that the odds that the fat percentage had increased were 47:1, while in the case of the 10 g. dosage group the small increase was not statistically significant. In the first week of the final control period the fat percentage of the 50 g. dosage group was 0.61 higher than expectation, this being statistically significant at odds of 237:1. This test coincided with the few days in which the yield remained elevated following the cessation of treatment. The fat percentage of the cows in the 10 g. dosage group showed no significant change. Subsequently there was a slight fall in the fat percentage of the 50 g. dosage group, but even in the last week of the final control period the fat percentage was still elevated. This fall in the fat percentage from the first to the last week of the final control period was statistically significant at odds of 15:1. It is thus apparent that iodinated Ardein fed by mouth increases the fat percentage in a similar manner to dried thyroid gland or thyroxine [Graham, 1934*a*; Herman *et al.* 1938].

The mean solids-not-fat percentage was determined from the total solids analyses, and the mean data are shown in Table 4.

Table 4. *Mean solids-not-fat percentage*

Period	...	Initial Mean of	Experimental			Final		
Week	...	3 weeks	1	2	3	1	2	3
Control		8.69	8.77	8.69	8.68	8.66	8.71	8.74
50 g. dose		8.65	8.72	8.66	8.74	8.50	8.80	8.70
10 g. dose		8.59	8.76	8.65	8.56	8.46	8.17	8.64

The changes in the solids-not-fat percentage during the experimental period were very variable, and although there was an apparent increase in the solids-not-fat percentage of 0.10 during the last week of treatment in the case of those cows which received 50 g. of iodinated protein, statistical analysis showed that this was not significant. A variability in the response of the solids-not-fat percentage to thyroxine injections has been reported by the Missouri workers [Ralston, Cowser, Ragsdale, Herman & Turner, 1940].

The increase in the fat percentage indicates that the yield of fat per day increased to an even greater extent than milk production. Table 5 shows the mean weekly yields of fat for the three treatment groups.

Table 5. *Mean fat yields in pounds per week*

Period	...	Initial	Experimental			Final		
Week	...	Mean of 3 weeks	1	2	3	1	2	3
Control		7.08	6.66	6.75	6.13	6.14	6.02	5.73
50 g. dose		6.80	7.10	8.72	8.51	8.00	5.25	5.37
10 g. dose		6.40	5.80	5.39	5.07	4.36	3.93	3.35
Response to 50 g. (lb.)			+0.72	+2.25	+2.66	+2.16	-0.49	-0.08
Response to 10 g. (lb.)			-0.18	-0.68	-0.38	-1.10	-1.41	-1.70

As expected, there was a considerable increase in the weight of fat secreted, when 50 g. of iodinated Ardein N4MB were fed. This amounted to 2.66 lb. per week during the last week of treatment—an increase of nearly 40% which was very highly significant statistically. This indicates that as in the case of cows injected with thyroxine, iodinated Ardein exerts a specific effect on fat production as well as on milk production. The depression occurring in the yields of the cows in the 10 g. dosage group was not significant, and inspection of the individual yields showed that the depression was again largely due to cow P19.

In a similar way it can be shown that the yield of solids-not-fat was increased during the experimental period when 50 g. of iodinated Ardein were fed.

Because of the large quantity of iodine ingested by the cows, composite samples of milk were taken on the 19th day of treatment, and the iodine content was kindly determined by Miss Simpson of the Rowett Research Institute at Aberdeen. Previous tests had shown that the iodine content of normal milk could be regarded as negligible, so only the milk from the two groups which received the iodinated Ardein was analysed. The iodine contents of the two milk samples were 204 and 84 µg. per 100 ml. in the 50 and 10 g. dose groups.

There was thus a considerable excretion of iodine in the milk in both groups, but although the form in which the iodine was present in the milk was not known, a number of palatability tests revealed no abnormality in the flavour of the milk. More recent experiments by Robertson [1945] have shown that milk produced by cows treated with iodinated protein has no abnormal physiological effect in humans.

#### *Physiological effects*

The effect of thyroid feeding or thyroxine injection on the normal metabolism of the dairy cow, as judged by heart rates, respiration rates, body temperatures, blood composition, and body weights, has been studied by some of the workers whose results have been briefly reviewed above. It was therefore felt desirable to collect

similar data for cows fed iodinated proteins, and, at the same time, to be able to form an accurate opinion on the effect of iodinated protein stimulation on the well-being of the cow. The records collected were limited to those which did not upset the animals unduly, and were supplemented by careful observation of each cow on each day of the experiment.

Heart rates were taken twice daily on 6 days of each week throughout the experiment, using a technique which has been described previously [Blaxter, 1943]. The records were taken at 7 a.m. and at 4 p.m., and any abnormal rates were always checked. Table 6 and Fig. 2 show the mean heart rates during the whole experiment.

Table 6. *Mean heart rates of the cows in beats per minute*

Period	...	Initial			Experimental			Final		
Week	...	1	2	3	1	2	3	1	2	3
Controls		62.3	60.5	57.6	57.3	56.5	56.1	52.9	51.8	50.9
50 g. dose		59.5	57.6	56.3	63.8	74.8	78.5	68.2	57.2	58.1
10 g. dose		66.5	61.1	63.6	63.6	61.4	62.6	59.8	57.2	57.9
Response to 50 g.					+8.7	+20.6	+24.7	+17.6	+7.7	+9.5
Response to 10 g.					+2.7	+1.3	+2.9	+3.3	+1.8	+3.4

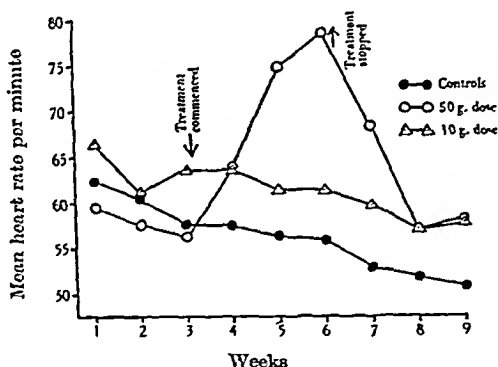


FIG. 2. The effect on the heart rate of feeding iodinated Ardein N4MB for a period of 3 weeks.

The heart rates of those cows which received 50 g. of iodinated Ardein N4MB increased very considerably. The mean increase during the three experimental weeks was 18.1 beats per minute and was very highly significant statistically (odds greater than 999:1). The maximum increase occurred during the 20th day of treatment when it was 25.2 beats per minute or 43.5%. There was no statistically significant change in the heart rates of the cows which received 10 g. of iodinated Ardein N4MB. The evening heart rates, which are normally higher than the morning rates, were analysed separately and there was found to be no difference between the increases in the morning and the evening heart rates in relation to treatment. During the final control period there was a depression of the heart rates of those cows which received the 50 g. dose, but the rates did not fall to the comparable level which was reached by the control cows, even at the end of the period. This tends to suggest a long-term effect of thyroxine stimulation on the heart rate, but it has been observed that pregnancy and nutrition exert a considerable effect on



the normal rate [Blaxter, 1944], and one cow was affected by a slight chill during the last week of the final period which resulted in an elevated heart rate. Although the difference between the control cows and those which had received the 50 g. dose was statistically significant, further data are required to confirm whether iodinated protein stimulation has a prolonged effect on the heart rate.

Respiration rates were recorded twice daily for 6 days in each week throughout the experiment. The record was made immediately following the heart-rate recording. The method used was to count the number of flank movements occurring in 1 min., taking care not to confuse them with rumen contractions. There was found to be a considerable variation in this rate over short periods. In spite of this the data in Table 7 indicate that iodinated Ardein N4MB exerted an effect on the respiration rate.

Table 7. *Mean number of respirations per minute*

Period	...	Preliminary			Experimental			Final		
Week	...	1	2	3	1	2	3	1	2	3
Control		35.1	37.4	29.7	30.0	31.1	33.3	31.4	30.8	34.2
50 g. dose		33.6	35.3	30.0	34.8	38.6	38.9	35.9	29.3	32.0
10 g. dose		36.1	38.3	31.1	32.2	32.2	31.2	30.7	28.7	31.4
Response of 50 g. group					+5.9	+8.6	+6.7	+5.6	-0.4	-1.1
Response of 10 g. group					+1.1	+0.1	-3.1	-1.7	-3.1	-3.8

The considerable variation from week to week was largely due to variation in the byre temperature, but there was a large increase of 6.7 respirations per minute or 20.4 % during the entire experimental period in the case of the cows which received the 50 g. dose, whereas a slight but not statistically significant fall occurred in the respiration rate of those cows which received 10 g. There was a fall of the respiration rate of the cows which received 50 g. of iodinated Ardein during the final control period, back to a normal value. The maximum response occurred on the 12th day of experiment in the case of these cows when it amounted to +14.0 respirations per minute or +42.7 %. At this time environmental temperature was high. Because of this variation in the response in the respiration rate with cowshed conditions, the mean daily response of those cows which received the 50 g. dose was correlated with the mean dry-bulb temperature of the cowshed. The correlation ( $r$ ) was +0.629 statistically significantly different from zero correlation at odds of 33:1, and a regression analysis indicated that besides the normal increase in rate with environmental temperature, the response of treated cows increased 0.5 respirations per minute for every degree Fahrenheit rise in the cowshed temperature. This shows that when environmental temperature is high the response in the respiration rate will be accentuated. This may be of importance in the use of iodinated protein in the summer months, for it indicates the cow's difficulty in heat disposal when her metabolism is increased and environmental temperature is high.

The rectal temperature of each cow was taken twice daily throughout the experiment. Care was taken to ensure that the bulb of the clinical thermometer was in close contact with the rectal wall, and any aberrant values were checked. The 30 sec. thermometer was held in position for 1 min. Although there was a small increase in the rectal temperature of the cows which received the 50 g. dose, there was nothing to suggest that the temperature regulating mechanism of the cows was

unduly disturbed, and in view of the smallness of the change it is doubtful whether it was of any major significance.

The cows were weighed on single days throughout the experiment, and during the preliminary control period and the first week of the final control period, 3-day weighings were carried out. The latter have been used to calculate the changes in body weight occurring during the experiment, and are shown in Table 8.

Table 8. *Mean changes in body weight in lb. from the preliminary control period to the first week of the final control period*

Treatment	Actual gain or loss	Net gain or loss
Control	+28.6	—
50 g. dose	-56.3	-84.9
10 g. dose	+46.7	+18.1

The net losses in weight showed that the cows fed 50 g. of iodinated Ardein N4MB lost considerable body weight (statistically significant at odds of 356:1), but the net increase in weight of the 10 g. group was not statistically significant. The daily weighings indicated that the loss of weight was largely concentrated in the second week of treatment when the net loss was 52.2 lb. in 7 days. Following the cessation of treatment there was an extremely rapid gain in live weight by the cows in the 50 g. dosage group at the rate of 40.7 lb. from the 4th to the 11th day of the final period. The gain was then much smaller, being only 4.4 lb. during the seven subsequent days. During the final period the increase in live weight of the treated cows was significantly greater than that of the control cows at odds of 32:1. The cows in the 10 g. dosage group also increased in body weight during this period but this increase was not statistically significant. The rapidity of the onset of the loss of weight and the rapid gain when treatment stopped, suggests that the loss may have been a result of a change in the amount of fill of the rumen and intestine, or that a slight tissue dehydration may have taken place. At the end of treatment, however, it was quite evident that the cows had lost both condition and flesh, this being confirmed by three independent observers.

When it was found that considerable increases in the metabolism of the cows had taken place blood samples were taken and Dr Green of the Ministry of Agriculture's Veterinary Laboratories at Weybridge carried out complete analyses. It was possible to transport the blood from Shinfield to Weybridge within 3 hr. and thus to prevent undesirable changes occurring. Unfortunately, blood analyses were not made in the control period, so that no control of the individual cow variation

Table 9. *Composition of venous (jugular) blood*

Treatment	Serum			Whole blood			Plasma CO <sub>2</sub> com- bining capacity vol./ 100 ml.
	Calcium mg./ 100 ml.	Mag- nesium mg./ 100 ml.	Acetone mg./ 100 ml.	Haemo- globin g./ 100 ml.	Sugar mg./ 100 ml.	Inor- ganic P mg./ 100 ml.	
Control	10.55	2.35	3.40	9.80	60.5	4.27	66.0
50 g. dose	10.20	2.35	2.70	10.00	74.0	3.95	53.7
10 g. dose	10.22	2.32	2.50	9.75	60.0	3.55	63.4
Significant difference <i>P</i> = 0.05	0.62	0.13	0.98	1.98	7.25	0.88	11.4

was possible. The data in Table 9 refer to samples of venous (jugular) blood taken during the morning of the 19th day of experimental treatment. Differences that would be significant where  $P = 0.05$  are given in the table for comparison with the observed differences. The treatment means for serum calcium, serum magnesium and serum acetone showed no differences which could be ascribed to differences in treatment, and all values were within the normal range [Green, 1943]. Neither haemoglobin nor inorganic phosphorus showed any change from a normal value. The blood sugar, however, was much greater for those cows which received the 50 g. dose than for those which received the 10 g. dose or the controls, and this difference of 23.3% was highly significant statistically. There was no indication of any abnormality in those cows which received the dose of 10 g. The plasma alkali reserve was lower for the cows in the 50 g. dosage group, each cow in the group having a lower value than her corresponding control cow. Variation in the 10 g. dosage group, however, reduces the reliance which can be placed in these means and it is therefore improbable that any change occurred. It can therefore be concluded that nothing abnormal was shown by the chemistry of the blood of the cows with experimental hypermetabolism which would indicate an impairment of vital functions or a metabolic disorder. The only exception was the elevated blood sugar, a change which is known to occur in experimental hyperthyroidism.

It will be remembered that one of the most disturbing features of the earlier experiments conducted by the Iodinated Protein Group, was the rapid onset of symptoms of iodine poisoning when iodinated proteins were fed. The total intake of iodine by cows receiving the 50 g. dose of iodinated Ardein N4MB was 2.9 g. per day, distributed equally in two meals. Careful observations were made for symptoms of iodism, or symptoms likely to be confused with iodism, but no such symptoms occurred.

On each day of the experiment each animal was carefully inspected and notes of any abnormality were taken. Most of these abnormalities were only minute differences from the normal. The observations made during the initial period were collected more for the appraisal of the norm for each cow than as a record of abnormalities. These records apply to only four cows in each treatment group and accurate decisions from such highly subjective observations are difficult to make. The data are presented under treatment headings.

(a) *Control cows.* Except for a slight loosening of the faeces of two cows and a slight cold affecting another for a few days, no abnormalities occurred in this group.

(b) *10 g. dosage group.* One cow (P19) in this group sweated badly on occasions on the warmer days of the initial control period and again during the experimental period. The same cow dropped in yield considerably—far more rapidly than was expected—during the whole period of the experiment, and, in the last week of experiment she secreted milk with a 'lipase flavour'. Cow L15 had a muco-purulent discharge from the vulva during one day of the preliminary control period, and although thought in-calf she returned to service a few weeks later.

(c) *50 g. dosage group.* The elevated metabolism of the cows which received this dose was reflected in many ways. The intensity of the heart sounds increased considerably when the increase in rate became apparent. Examination of the mucous membranes of the vagina revealed some slight vasodilatation. These data tend to

indicate a considerable increase in heart output and when treatment stopped there was difficulty in acclimatizing the circulation to the changed metabolism. Sweating was observed in three of the cows (P7, C8, and F31), no sweating having been observed previously. The remaining cow (D) did not sweat, probably because of her breed—a Guernsey. Nervous effects were also noticeable. Two cows, C8 and D, became irritable during the experimental period, especially so during the heart-rate recording. In the case of cow C8 this was so pronounced that it was remarked on by the milker. Cow F31 showed a muscular tremor and twitching on two consecutive days of the 2nd experimental week, but this was not observed again. A distinctly tiring effect of the dose was noted, for three of the cows persisted in lying down during the milking period, a practice which is unusual in the Shinfield herd and was not observed in any of the other cows. One further general point was observed by the cowman and those in close contact with the animals, and that was that a distinct swelling of the udder coincided with the period of increased milk production.

Besides these observations dealing with the effect of treatment, three of the cows were abnormal but not as a result of treatment. Cow F31, a cow known to excrete haemolytic streptococci in her milk and to have fibrosis of the udder, secreted milk with clots on one day of the preliminary period. Subsequently her behaviour in this respect was quite normal. Cow D, a Guernsey, had a slight chill in her left lung during the last week of the final control period. This lasted for 3 days, and was accompanied by an increase in both temperature and heart rate, the latter largely accounting for the elevated mean heart rates during this period (see Table 6). Cow C8, on the morning of the 12th day of treatment, had an almost complete paralysis of the right ear, very profuse salivation, a swelling over the right eye and refused her food. Treatment was continued, she was persuaded to eat her food and recovered while treatment was still taking place. A detailed veterinary examination of this cow was made by Prof. T. Dalling and Mr A. T. Cowie, and following further observation, it was agreed that her condition was due to a blow over the eye, affecting the nerve supply to the salivary glands and ear. In view of the increased nervousness and irritability of this cow this seems the correct explanation.

### *Discussion*

The results of the experiment indicate that a pronounced increase in both milk production and milk-fat production occurred when 50 g. of iodinated Ardein N4MB were fed to four dairy cows, while when a dose of 10 g. was given there was little indication of any response, although this may have been due to the small number of animals available and to the abnormal behaviour of cow P19. The clinical data, however, do not indicate any marked change in the metabolism of the cows in this group. In the 50 g. dosage group marked changes in metabolism occurred, and together with the increase in milk and fat production show that the effect of feeding an iodinated protein is similar in all respects to the effects of feeding dried thyroid gland or the injection of the pure hormone. That the effect was due to additional protein alone is discounted by the inclusion of pure Ardein in the ration of the control cows.

## SECOND EXPERIMENT WITH IODINATED ARDEIN N4MB (Exp. 5)

Although increases in production occurred in the previous experiment, there were undesirable features associated with this stimulation, and these were all symptoms associated with hypermetabolism—a greatly stimulated heart rate, increased respiration rate and rectal temperature, together with a severe loss of body weight. Treatment lasted for 3 weeks, and although it appeared that a maximum level of metabolism had been reached, it was obviously of importance to find the effect of a prolonged dosage on the health of the dairy cow. It was therefore decided to carry out a further experiment with iodinated Ardein N4MB with three main objects: firstly, to confirm the results of the previous experiment; secondly, to collect as much additional information as possible on the effect of iodinated protein on the health of the cow and on milk composition; and lastly, to find the effect of continued dosage on the well-being of the cow.

*Plan of experiment*

The supply of iodinated Ardein N4MB was small, and as the element of risk was considered high the experiment was conducted with only four cows, two of which received treatment. A further two cows were included in the experiment to assay a further batch of iodinated Ardein of doubtful potency from the chemical standpoint. The results of this assay of iodinated Ardeins N9+10 are not included in this paper. Details of the experimental cows are given in Table 10.

Table 10. *Details of experimental cows*

Cow	No. of times calved	Date of last calving	Date due to calve	Date of next calving	Mean daily yield lb.	Treatment allocation
L13	3	13 Jan. 1942	Not served	Slaughtered	16.8	Control
L7	3	7 Mar. 1942	Not served	Slaughtered	16.2	50 g.
F48	4	6 July 1942	11 July 1943	13 July 1943	16.2	Control
P20	2	17 July 1942	6 Sept. 1943	4 Sept. 1943	19.2	50 g.

Cows L7 and F48 had been control cows in the previous experiment, and cow P20 had received the 10 g. dose without showing any effect. Cow L13 had not previously been used for experimental purposes. The two treatments were as follows.

*Controls:* normal winter rations with the addition of 100 g. of dried yeast and 50 g. of pure Ardein.

*50 g. dose:* normal winter rations with the addition of 100 g. of dried yeast and 50 g. of iodinated Ardein N4MB.

The normal winter rations consisted of 13 lb. of meadow hay, 65 lb. of a mixture of mangels and chaff, 4 lb. of oat straw and 6 lb. of concentrates. During the final control period the mangels and chaff mixture was changed, owing to failure of the mangel supplies, to an amount of potatoes equivalent on a starch basis. The concentrate mixture was made bulky by the inclusion of oats and sugar-beet pulp and was ground to facilitate the admixture of the iodinated Ardein. The object of feeding the dried yeast mixed with the cows' concentrates was to avoid refusals of food, for the strong smell of the yeast disguised the smell of the iodinated Ardein, and the cows had been accustomed to its strong taste before the experiment. On each day of the experiment the cows were allowed out for a few hours on to a bare pasture

for exercise, and, in the latter part of the experimental period, small amounts of grass were available, and were eaten during exercising time. The experiment was of 84 days duration, commencing with an initial period of 14 days when only the basal winter ration was fed, and including an experimental period of 49 days, and a final control period of 21 days. Treatment commenced on 1 March 1943.

### *Results*

A larger number of records were taken from these four cows than from those in the previous experiment and far greater attention was paid to detail. There were, however, only two treated animals and two controls, so that a statistical analysis to determine the validity of the conclusions on the basis of internal evidence alone has not been made. As in the previous experiment the results have been expressed in terms of 'responses', but attention is largely given to individual changes in the factors studied in relation to the range of variability which was normally to be expected. The calculation of a response in milk production, or in any of the variables studied assumes that the experimental animals if they had received no treatment would have reacted in exactly the same way as the control animals. This is a safe assumption to make when large numbers of animals are used. With only two replications, there is a danger that the mean response will not be accurate and the inaccuracy of the mean response increases with the length of the experimental period. Part of this inaccuracy can be obviated by a calculation of the 'expected yields', using the values in the initial and final periods for calculating a regression and interpolating. This equates for any differential rate of normal decline in the yield of the control cows, compared with the experimental cows. This is only a legitimate method when treatment has no 'carry-over' effect elevating or depressing the values in the final control period. As this is not known in the case of iodinated-protein-treated animals, the 'response' method of calculation has been used, and the assumptions involved are taken fully into account.

### *Palatability*

Cow L7 would not eat her food at the commencement of treatment but was eventually persuaded to do so without any of the usual artifices. Later, after 40 days of treatment, she again refused some of her concentrate + iodinated Ardein mixture, but was only off feed for 1 day. Cow P20 did not refuse food on any occasion.

### *The effect on milk production and composition*

Table 11 shows the mean weekly yields of both fat and milk by the two groups of cows and the calculated responses. These data are shown graphically in Figs. 3 and 4.

There was a very large increase in milk secretion, the maximum increase occurring in the third week of treatment, when the cows which received the iodinated Ardein were producing 6.2 lb. more milk per day. The yields, however, declined following the third week of treatment, but there was still a considerable elevation of yield which persisted until treatment stopped. Then, following an elevation of 4 days, milk yields dropped precipitously, there being an indication of a recovery in the third week of the final control period. Milk-fat production was increased to a far greater extent, and the same cycle of changes occurred, the final recovery being less conspicuous.

Table 11. *Mean weekly yields of fat and milk in pounds*

Factor	Treatment	Initial period (weeks)		Experimental period (weeks)							Final period (weeks)		
		1	2	1	2	3	4	5	6	7	1	2	3
Milk yield	Controls	114.6	116.3	121.3	116.3	114.6	105.2	108.8	106.6	106.3	103.6	103.4	94.7
	50 g. dose	122.0	125.7	137.6	162.5	166.6	153.7	154.6	134.6	135.1	124.5	81.8	81.3
	Response in lb. % response			+7.8	+37.8	+43.6	+40.1	+37.4	+31.1	+19.0	+12.5	-30.0	-22.2
Fat yield	Controls	4.55	4.72	4.96	4.67	4.62	4.24	3.91	3.96	4.02	3.90	3.83	3.56
	50 g. dose	4.93	5.12	5.90	7.69	7.73	7.38	6.86	6.57	5.94	5.38	3.04	2.87
	Response in lb. % response			+0.54	+2.63	+2.71	+2.74	+2.56	+2.22	+1.53	+1.00	-1.18	-1.08
				+10.8	+52.3	+53.9	54.5	+50.9	+44.1	+30.4	+19.9	-23.5	-21.5

Table 12. *Percentage composition of the milk*

Constituent	Treatment	Initial period (weeks)		Experimental period (weeks)							Final period (weeks)		
		1	2	1	2	3	4	5	6	7	1	2	3
Fat %	Control	3.96	4.05	4.07	4.00	4.02	4.02	3.67	3.70	3.77	3.84	3.72	3.78
	50 g. dose	4.06	4.08	4.29	4.75	4.64	4.81	4.44	4.50	4.42	4.33	3.73	3.50
	Response (% units) % response			+0.16	+0.69	+0.56	+0.73	+0.71	+0.74	+0.50	+0.43	-0.05	-0.32
Solids-not-fat %	Control	8.75	8.56	8.76	8.67	8.68	8.61	8.65	8.69	8.75	8.80	8.88	8.89
	50 g. dose	8.76	8.61	8.75	8.74	8.82	8.77	8.73	8.83	8.76	8.84	8.79	8.94
	Response (% units) % response			-0.04	+0.03	+0.11	+0.13	+0.05	+0.08	-0.02	0.0	-0.13	+0.02
Solids-not-fat in fat-free milk %	Control	9.11	8.93	9.13	9.03	9.04	8.87	8.88	8.91	9.09	9.16	9.27	9.24
	50 g. dose	9.13	8.98	9.14	9.17	9.25	9.22	9.14	9.24	9.16	9.40	9.13	9.26
	Response (% units) % response			-0.01	+0.11	+0.18	+0.32	+0.23	+0.30	+0.04	+0.21	-0.17	-0.04
				-0.2	+1.2	+2.0	+3.5	+2.5	+3.3	+0.5	+2.3	-1.9	-0.4

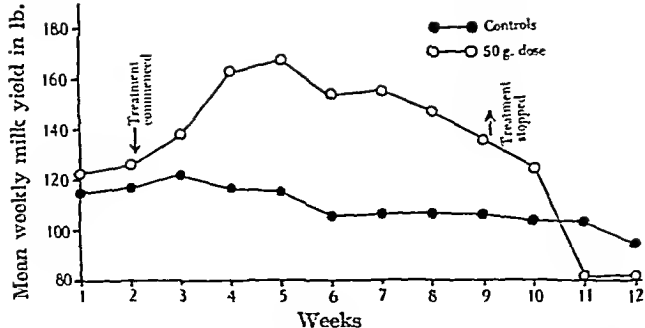


FIG. 3. The effect on milk yield of feeding iodinated Ardein N4MB for a period of 7 weeks.

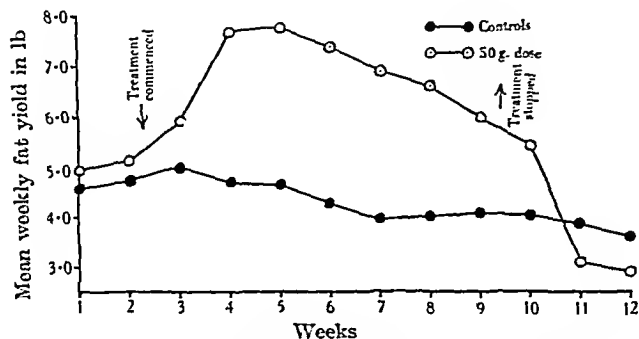


FIG. 4. The effect on the yield of milk fat of feeding iodinated Ardein N4MB for a period of 7 weeks.

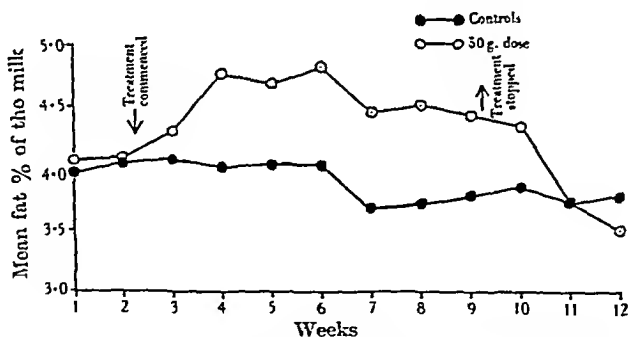


FIG. 5. The effect on the fat percentage of the milk of feeding iodinated Ardein N4MB for a period of 7 weeks.

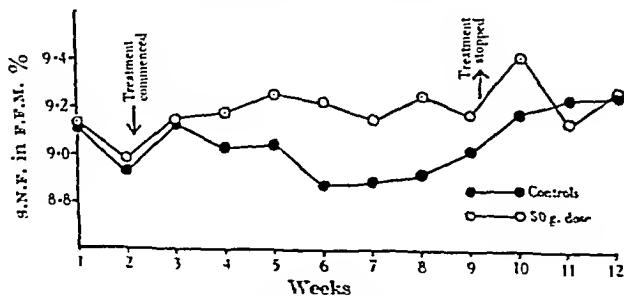


FIG. 6. The effect on the percentage of solids-not-fat in the fat-free milk of feeding iodinated Ardein N4MB for a period of 7 weeks.



Table 12 shows the data which were collected on the composition of the milk, and these are also shown in Figs. 5 and 6.

It is evident that there was an appreciable increase in the fat percentage of the milk during the period of treatment, and a decided fall when treatment stopped. Similarly, the solids-not-fat percentage increased slightly during treatment but not to any appreciable extent. That there was an increase in the solids-not-fat percentage is indicated, however, by the slight depression in the post-experimental period. Nevertheless, the increase was far lower than that reported by Folley & White [1936] and of the same order as that reported by other workers who have used thyroxine [Herman *et al.* 1938; Ralston *et al.* 1940]. This is probably a result of the method of calculation used, for Folley & White calculated the solids-not-fat as a percentage of the fat-free milk, using the method suggested by Bartlett [1934], thus correcting for any concomitant change in the percentage of fat, whereas other workers have used uncorrected data. The percentage of solids-not-fat in the fat-free milk is shown at the bottom of Table 12 and in Fig. 6. These show that there was a decided increase in the solids-not-fat content of the milk when the calculation is made in this way, and indeed approaches that found by Folley & White. Such an increase in the solids-not-fat content was anticipated and Dr S. J. Rowland kindly carried out determinations of lactose and total N in composite milk samples from each cow at weekly intervals. The very small changes which were found appeared more of the nature of normal fluctuations rather than indications of a treatment effect. In view of the small number of animals involved, no conclusions have been drawn. Freezing-point determinations on the milks carried out at intervals showed no departure from the normal range of variability.

### Physiological effects

Heart rates, respiration rates, and rectal temperatures were recorded twice daily at 7.30 a.m. and 4.30 p.m. on 6 days of each week of the experiment. The heart rates and respiration rates are summarized in Table 13.

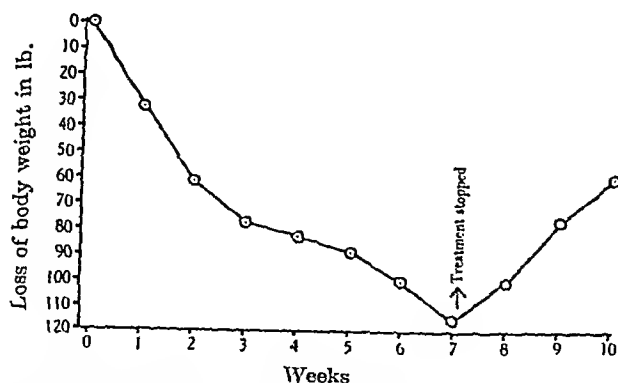


FIG. 7. The effect of feeding iodinated Ardein N4MB for a period of 7 weeks on body weight, and the recovery of body weight when treatment stopped.

Both the heart rates and the respiration rates of the cows increased when 50 g. of iodinated Ardein N4MB were fed, both reaching maxima by the 3rd week of treatment and subsequently declining. When treatment stopped, a decided fall in the

Table 13. Heart rates and respiration rates per minute

Rate	Treatment	Initial period (weeks)			Experimental period (weeks)							Final period (weeks)		
		1	2		1	2	3	4	5	6	7	1	2	3
Heart	Control	57.5	58.3		58.0	58.1	57.8	56.5	56.1	58.3	56.3	63.4	63.2	65.7
	50 g. dose	53.3	55.6		58.1	74.7	75.0	72.4	70.6	72.7	70.3	67.8	62.2	61.3
	Response—beats/min. % response				+3.9	+20.3	+21.7	+19.6	+18.2	+18.1	+14.7	+9.1	+2.6	-0.8
Respiration	Control	31.8	30.3		+7.2	+37.5	+40.1	+36.2	+33.6	+33.5	+27.1	+10.8	+4.8	-1.5
	50 g. dose	26.1	24.6		33.3	30.0	33.5	34.6	31.1	34.0	36.0	29.0	31.7	33.8
	Response—respirations/min. % response				36.4	38.2	40.5	38.6	30.2	37.0	34.7	35.4	27.8	28.7
					+2.0	+13.7	+12.8	+9.7	+10.8	+7.7	+3.5	+12.1	+1.8	+0.8
					+11.4	+53.8	+50.1	+38.0	+42.1	+30.5	+13.6	+47.0	+7.2	+3.3

Table 14. Live weights of cows in pounds at weekly intervals and losses in weight calculated from three weightings each week

Treatment	Initial period	Experimental period (weeks)							Final period (weeks)		
		1	2	3	4	5	6	7	1	2	3
Control	1310.3	1311.8	1315.2	1318.2	1312.7	1321.0	1310.6	1312.1	1308.8	1320.8	1328.6
50 g. dose	1288.7	1257.6	1227.5	1211.0	1198.6	1198.7	1184.5	1162.8	1180.5	1223.6	1251.0
Loss of weight		-32.2	-61.0	-77.4	-82.3	-88.4	-104.2	-115.5	—	—	—

heart rates occurred and the rates had returned to normal by the 2nd week of the final period. Respiration rates, however, did not return to normal for a considerable time, largely due to an environmental temperature change.

The rectal temperatures of the cows have been used as an indication of their normality, and cow L7 was the only one showing any serious change. Her average body temperature, measured at wide intervals during several years, had been low as judged by other cows and was low ( $100\text{--}65^{\circ}\text{F.}$ ) in the initial period of the experiment. In the 2nd week of treatment her temperature rose to  $101\cdot9^{\circ}\text{F.}$  and subsequently dropped to a value varying between  $101\cdot0$  and  $101\cdot4^{\circ}\text{F.}$  Except for a stiffness at the joints which was normal for this cow, no cause of this could be ascertained other than the increased metabolism, and it appears probable that it was indeed a treatment effect. In the 2nd week of the final period her temperature again increased owing to a mastitis outbreak. Cow P20 did not show any abnormality save an increase of  $0\cdot5^{\circ}\text{F.}$  during the 6th week of treatment, the cause of which could not be ascertained. The control animals behaved normally except that the rectal temperature of cow F48 increased  $0\cdot8^{\circ}\text{F.}$  over the 12 weeks of experiment, a change probably related to pregnancy.

The cows also lost weight during the treatment period. The live weights of the cows at weekly intervals are shown in Table 14. The net loss in body weight and its subsequent recovery are shown in Fig. 7.

The loss of weight as given makes allowance for the slight changes in weight of the control animals. Otherwise the losses in weight during the whole period were cow P20 118·0 lb. and cow L7 133·8 lb. Subsequently the weight recoveries were cow P20 +112·4 lb. and cow L7 +71·0 lb. in the 3-week period. This loss in weight, which is shown graphically in Fig. 7, was accompanied by a loss of condition as judged by eye. This was apparent on the 11th day of treatment and both cows continued to lose condition until treatment stopped. The loss of fat over the ribs and over the back was very noticeable in the case of cow L7, and during the last 2 or 3 weeks of treatment the thigh muscles began to lose their fullness in the case of this cow. The cows did not reach an emaciated state, but were definitely poorer than the comparable control cows.

In view of the slight changes in blood composition which had been found in the previous experiment samples of venous blood (jugular) were again sent to Dr H. H. Green, who kindly carried out complete analyses. These were taken once during the initial period, once after 3 weeks' treatment, and again in the last week of treatment. In the case of the cows fed iodinated Ardein the blood serum protein, calculated from the refractive index of the serum, showed that there was no evidence of a dehydration or anhydraemia, and this was confirmed by the absence of specific changes in the haemoglobin. Small changes in the blood serum calcium occurred—probably associated with slight changes in the inorganic phosphorus. Although there was no conclusive evidence of a change in the serum calcium this does not necessarily mean that no depletion of the body reserves of calcium occurred. The net rise in the blood sugar was small, amounting to only 7%. There was no indication of ketosis, raised serum magnesium, or any secondary anaemia, conditions which have been noted in the hyperthyroid human [Peters & van Slyke, 1931], and thus the blood chemistry of the individuals fed 50 g. of iodinated Ardein indicates no departure from the normal.

Because of the specific effect of thyroxine on the phosphatase of the milk (phosphomonomesterase A<sub>1</sub> [Folley & Kay, 1936]) [Folley & White, 1936], Dr S. J. Folley determined the phosphatase of both plasma and milk on the same occasions as the determinations of blood constituents were made. The plasma phosphatase showed much variation, and no conclusions could be drawn, but the effect on the milk phosphatase was striking. The mean corrected decrease was 111.4 King & Armstrong units per 100 ml. in the 3rd week of experiment and 141.6 units in the 7th week, changes of great magnitude confirming that the effect of iodinated Ardein is a thyroxine-like effect. The data on blood composition and the phosphatase of the milk are shown in Table 15.

Table 15. *Blood composition and milk phosphatase*

Determination	Week	Control cows		50 g. dose cows	
		L 13	F 48	L 7	P 20
Serum protein, %	Control	8.10	7.75	6.95	6.60
	3	7.90	7.65	6.95	6.65
	7	7.90	7.50	6.80	6.55
Serum Ca, mg./100 ml.	Control	10.4	10.0	10.3	10.4
	3	9.9	9.9	9.9	9.7
	7	10.4	9.8	10.4	10.7
Serum Mg, mg./100 ml.	Control	2.3	2.3	2.3	2.5
	3	2.2	2.2	2.2	2.3
	7	2.1	2.2	2.3	2.3
Serum ketones, mg./100 ml.	Control	3.7	4.0	4.8	4.6
	3	4.8	4.0	3.7	5.2
	7	3.7	3.5	3.5	3.5
Blood sugar, mg./100 ml.	Control	53	51	51	55
	3	54	56	59	63
	7	57	58	60	64
Blood inorganic P, mg./100 ml.	Control	3.5	4.0	4.6	4.2
	3	3.2	3.8	4.8	4.6
	7	3.4	4.0	4.2	4.8
Haemoglobin, g./100 ml.	Control	10.7	10.4	11.4	10.2
	3	11.1	10.9	11.8	10.4
	7	10.4	10.6	11.4	11.1
Plasma CO <sub>2</sub> combining capacity, vol. 100 ml.	Control	67	65	71	67
	3	67	67	72	69
	7	53	65	56	69
Plasma phosphatase, King & Armstrong units/100 ml.	Control	10.4	8.6	50.6	13.6
	3	10.5	6.0	43.3	16.5
	7	15.4	6.4	55.6	20.9
Milk phosphatase, King & Armstrong units/100 ml.	Control	261.6	127.7	159.3	156.4
	3	268.5	126.8	39.4	58.5
	7	241.4	210.8	51.7	42.6

Records were collected of any abnormalities noted. These are summarized under four headings: iodism symptoms, heart and circulatory abnormalities, nervous abnormalities, and other abnormalities.

*Iodism symptoms.* Cow L7 began salivating slightly on the 11th day of treatment and again on the 13th day. On the 17th day she had a slight sore place  $\frac{1}{2}$  in. in diameter in her right nostril and exuded a clear nasal mucus. The sore place had healed by the 22nd day. These very slight symptoms of iodism did not increase;

in fact they tended to disappear even though treatment continued. Cow P20 was not affected by iodism and despite continued dosage skin symptoms of iodism never occurred in either of the cows.

*Heart and circulatory abnormalities.* The heart sounds of cow L7 became abnormal on the 8th day of treatment. This cow normally had a low heart rate and by the 15th day the heart contractions were very irregular. This was again apparent on the 16th day when a more detailed examination was made. It appeared that the abnormality was an extra-systolic contraction interpolated between the normal rhythmical contraction of the heart muscle, and was probably a nervous effect, the result of the inability of the cow to accommodate her circulation rate to her greatly increased bodily metabolism. At the same time, by careful listening, it appeared that the valves of the heart were not closing the normal way, but although this had not been recorded in the initial period it was thought that this was not due to treatment but was an individual characteristic of the cow. The extra-systolic contraction increased in intensity until, on the 18th day, it was occurring once in every 5.6 sec. It was last heard on the 25th day of treatment and did not reappear. Valvular noises were heard again on the 30th day of treatment and during the final control period. Cow P20 showed no arrhythmia or abnormality of the heart saving the increased rate of beat. Following the commencement of the rise in heart rate the force of the beat increased in both cows, for the muscle sounds were augmented considerably.

*Nervous symptoms and changes in behaviour.* Cow P20 was always slightly irritable in the initial period, especially when her heart rate was being recorded. This irritability increased considerably during treatment and by the 18th day it was apparent that an increase in the nervousness of both the cows had occurred although no comment was made by the cowmen milking them. The hyper-metabolism tended to cause fatigue. Cow P20 commenced lying in the cowshed during the milking interval on the 12th day of treatment and again on the 14th and 15th days. While cow L7 did not lie in the cowshed, on the 44th day of treatment she was leaning heavily on the stanchion on her right side and had a disinclination to move. During the last 2 weeks of treatment there was a meagre supply of grass in the exercising paddock, and while both control cows spent some time grazing, both cows L7 and P20 spent part of their time lying and ruminating. Cow L7, during the last 2 weeks of treatment, became very nervous, this taking the form of flinching when she was approached, or when a noise was made. This was accentuated by touching her, but as far as could be ascertained it was not due to muscular pain. Both cows became less irritable in the final period. Sweating was not observed to any appreciable extent, slight signs of sweating during the night interval being apparent only in the first 2-3 weeks of treatment.

*Other abnormalities.* Several rectal temperature fluctuations have already been mentioned and in the majority of cases causes could be found. In the final control period the rectal temperature of cow L7 increased for several days owing to mastitis. For 2 days this could not be diagnosed as there was no clinical change in the udder, but on the 3rd day swelling of the affected quarter occurred and this was milked at 3-hourly intervals throughout the night. On the next day the cow's temperature again became normal.

On the 20th day of treatment, it appeared that P20 had developed a slight exophthalmos in the form of an increase of the palpebral fissure, but this observation is extremely subjective, and photographs of the cow were not sufficient to give an ample confirmation. The same cow scoured slightly throughout the treatment period and the faeces became harder when treatment stopped. The appetite of both cows increased, as judged by the time they took to consume their rations.

### GENERAL DISCUSSION

The short discussion of the results of the first experiment with iodinated Ardein N4MB showed that the effect of iodinated protein was similar to the effect of thyroxine injection in every way. This discussion deals with certain specific aspects of the second experiment, and the results of the first experiment are also included for comparison.

#### *Confirmation of the results of the first assay experiment*

The first 21 days of treatment in the two experiments are similar and it is of interest to compare the results obtained. This is shown in Table 16.

Table 16. Responses to 50 g. of iodinated Ardein N4MB

Factor measured	1st experiment		2nd experiment	
	Actual response	Percentage response	Actual response	Percentage response
Milk yield per day in 3rd week	+ 6.6 lb.	+ 29.3	+ 6.2 lb.	+ 35.2
Total increase in yield in 3 weeks	108.5 lb.	23.1	89.2 lb.	24.0
Heart rate per minute 3rd week	+ 24.7 beats	+ 42.7	+ 21.7 beats	+ 40.1
Respiration rate per minute 3rd week	+ 5.7 respirations	+ 17.3	+ 12.8 respirations	+ 50.1
Fat % in 3rd week	+ 0.37 % units	+ 8.5	+ 0.56 % units	+ 13.8
Fat yield in 3rd week	+ 2.66 lb.	+ 39.0	+ 2.71 lb.	+ 53.9
Net weight loss in 3 weeks	84.9 lb.	—	77.4 lb.	—
Solids-not-fat % in 3rd week (as determined)	+ 0.10 % units	+ 1.1	+ 0.11 % units	+ 1.3
Blood sugar in 3rd week	+ 13.5 mg./100 ml.	+ 22.5	+ 4.0 mg./100 ml.	+ 7.0
Rectal temperature mean increase in 3 weeks	0.147°F.	—	0.323°F.	—

The data show considerable agreement in response except in the case of body temperature, blood sugar, and respiratory rate. In the second experiment both the body temperature and the respiratory rate of the cows were elevated to a greater extent, and in this connexion it is of interest to note that a higher fat percentage was also evident. The respiration rate was higher largely due to a higher environmental temperature, and it should be remembered that a maximum respiration rate response of 14 per minute occurred in the first experiment when environmental temperature was high. The cause of the anomalous blood sugars is not known, but the error attached to the increases found in the first experiment is high owing to the absence of preliminary readings, both responses refer to jugular blood, and not to arterial blood, and in the second experiment a different feeding programme was in operation resulting in a totally different sampling of the blood in relation to the time of alimantation. It can therefore be stated that within the range of variability to be expected in the factors studied, the demonstrated changes due to iodinated Ardein feeding are readily reproducible.

*The evidence for a decline in response with continued treatment  
in the second experiment*

The tables showing the responses in milk production, fat production, milk composition, and metabolism indicate a falling off of the response in the 6th and 7th weeks of treatment, and subsequently a depression of milk and fat yield below the level of production of the control cows, apparent even in the 3rd week of the final period. Several factors have to be considered in this respect. First, the effect may be an error of extrapolation, for the response is based on the assumption that initially paired cows decline at the same rate; secondly, thyroxine treatment results in a temporary reduction of yield following the cessation of injections [Folley & White, 1936]; thirdly, cow L7 was affected by mastitis in the 2nd week of the final period, and mastitis depresses milk production; fourthly, cow L13, one of the control cows, did not decline in yield as expected but maintained her yield at a very constant level; and lastly, the small differences in behaviour at grass which were noted may have given a greater stimulus to the control cows. From these possible causes of the decline in response there is not sufficient evidence to suggest a diminution of the response as a result of the prolonged treatment, and although at the cessation of treatment a marked depression of yield occurred, this was well within the error range of an extrapolated initial yield.

*The effect of iodinated protein on the efficiency of food utilization*

In the second experiment, food intakes were maintained constant between the two groups of cows in order to make it possible to draw conclusions on the effect of treatment uncomplicated by any apparent over-feeding of the experimental cows. There was very slight over-feeding of both groups with respect to calories on the basis of their expected production without treatment. It is therefore possible to calculate the efficiency of the conversion of body tissue into milk in terms of energy. This has been done by using the responses for milk and fat production, calculated to a standard milk of 3.75% fat and the losses in live weight as determined. The mean energy value of 1 lb. of live-weight gain has been calculated from data of Weigner & Grandjean [1936] and the calorific value of the milk has been taken from Halnan's net energy figures [1929]. Efficiency therefore becomes the ratio of calories of milk energy/calories of live-weight energy  $\times 100$ . These data are shown in Table 17.

Table 17. *Calculated efficiency of conversion of live-weight energy  
to milk energy, as a percentage*

Week of feeding	Cumulative efficiency	Incremental or non-cumulative efficiency	Probable nature of weight loss
1	5.5	5.5	Ruminal and intestinal fill
2	17.1	29.7	Liver glycogen and easily mobilized fat
3	25.0	56.2	
4	34.4	183.1	Depot fat
5	41.5	137.9	
6	42.1	45.9	Body protein and muscle glycogen
7	42.4	44.2	

It will be noted that Table 17 is constructed on the assumption that one unit of live-weight loss has the same standard calorific value whatever loss occurs, but the incremental efficiencies show, as was indeed expected, that this is not correct. Although these efficiency ratios are subject to some considerable error, they tend to confirm that a decrease of fill, and perhaps a loss of body water, occur during at least the early stages of treatment. The changes in the ratio during the 7-week period probably indicate the nature of the reserves which are depleted when the metabolism of the cow is stimulated by feeding iodinated protein. In the case of laboratory animals it has been found that an initial effect of thyroid stimulation is a depletion of the liver glycogen reserves [Coggeshall & Green, 1933], and much later a breakdown of body protein and muscle glycogen occurs. As both carbohydrate and protein storage in the animal body is accompanied by storage of three or more parts of water whereas the storage of fat is accompanied by the storage of negligible quantities of water [Leathes & Raper, 1925], the probable nature of the reserves can be arrived at as shown in the last column of the table. This hypothesis accounts for the plateau of the live-weight loss curve and its subsequent steep downward trend, as shown in Fig. 7, but as this can only be ascertained by calorimetric experiment, the hypothesis can only be tentative. The figure usually accepted for the efficiency of the conversion of weight energy to milk energy is 60 %, and it is probable that the lower cumulative efficiency found is due to an increased basal metabolic rate, as well as to the inherent inaccuracy of the method used.

*The interpretation of the physiological data in relation  
to the health of the cows*

Feeding 50 g. of iodinated Ardein N4MB, while substantially increasing milk production, had a very pronounced effect on the normal physiological processes of the six cows involved. The most obvious was the loss of body weight, especially in the second experiment in which food was restricted in spite of the increased production, but changes in the resting heart rate, body temperature, and respiratory rate, though less recognizable at a cursory examination of the cows, were equally important. Coupled with these changes were the extra-systolic contraction of the heart of cow L7, muscular tremors and fatigue in cows F31, P20 and L7, nervous effects and changes in the normal behaviour of the cows. In general, however, the whole picture was not as severe as was expected, and, more important, no major symptoms occurred indicating permanent injury. In spite of this, at this high level of stimulation of the metabolism, the absence of a recognizable injury does not mean that the vital functions of the cow have remained unimpaired or that the productive life span has not been curtailed.

*The after-effects of treatment and the subsequent history of the cows*

Of the six cows which were treated with iodinated Ardein N4MB two were sold fat, and four subsequently calved again. Cows F31 and L7 were fattened very successfully during the spring and summer of 1943, and when they were slaughtered for beef no enlargements or obvious changes in the vital organs were seen. Their rate of gain of condition during fattening was very satisfactory. The remaining cows calved again, and the data on these cows are given in Table 18.



Table 18. *Subsequent history of treated cows P20, P7, D and C8 and control cows F48, O and P8*

Cow	Treatment	Date	Sex of calf	Normality of calf	Calving subsequent to treatment	
					Average daily yield in first 10 weeks of lactation lb.	Average daily yield in first 10 weeks of lactation prior to treatment lb.
P20	50 g. dose	4 Sept. 1943	B.C.	Normal	38.3	33.4
P7	"	7 June 1943	H.C. B.C. }	See text	35.1	33.0
D	"	25 Mar. 1943	B.C.	Normal	32.5	32.1
C8	"	9 June 1943	H.C.	Normal	35.0	36.1
F48	Control	13 July 1943	H.C.	Normal	33.5	31.0
O	"	3 July 1943	H.C.	Normal	33.3	33.6
P8	"	23 May 1943	H.C.	Normal	28.8*	41.9†

\* Very nervous and difficult to manage in this period and eventually sold fat.

† Yield at spring grass.

The data show that the yields of milk in the following lactation were quite within the normal range in the case of those cows which had been fed iodinated Ardein in the previous lactation.

All the calves born were quite normal with the exception of cow P7's twin calves. One, a bull, was born dead and the carcass was destroyed. The other, a heifer, survived but its development was abnormal in the pelvic region. It was sent to Mr C. W. Ottaway of the Royal Veterinary College for observation and dissection. Mr Ottaway was of the opinion that the defect—an incomplete development of the Müllerian duct—was hereditary in origin and was not due to the experimentally induced hypermetabolism during early pregnancy.

It is therefore apparent that those cows which had been treated with iodinated Ardein N4MB behaved in a quite normal manner and that their milk production was not impaired in the subsequent lactation.

### SUMMARY

1. Experiments in which iodinated proteins have been fed to milking cows are described.

2. Early experiments with iodinated proteins of low thyroidal activity resulted in little effect on milk production or metabolism, and iodism symptoms rapidly occurred in the experimental animals at the high doses used.

3. A highly active preparation, iodinated Ardein N4MB, stimulated milk production very considerably, and all available evidence showed a concomitant increase in the metabolism of the individual cows.

4. Even following a long period of treatment the evidence collected did not indicate that any permanent damage had been done to the cow.

My thanks are due to the many individuals who have helped and advised me in carrying out this work: to Dr S. J. Rowland and Mr A. Wagstaff for numerous determinations of milk fat and solids-not-fat; to Dr S. J. Folley for milk phosphatase

determinations; to Dr H. H. Green for blood analyses; to Miss B. Simpson for milk iodine determinations; to Mr Ottaway for his dissection of a calf and to Mr T. H. French and other members of the Dairy Husbandry Department of the National Institute for Research in Dairying for their co-operation at all times.

I would also like to express my gratitude to the members of the Iodinated Protein Group of the Agricultural Research Council, for the encouragement they have given, for the opportunities which they have afforded, and for their permission to publish these results.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 4. THE EFFECT OF IODINATED PROTEIN FEEDING ON THE LACTATING COW

### (ii) THE EFFECTS OF IODINATED CASEIN

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(Received 13 October 1944)

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### INTRODUCTION

In the preceding paper [Blaxter, 1945] it was shown that iodinated Ardein N4MB\* [Pitt Rivers & Randall, 1945] had a considerable effect on the milk production and metabolism of dairy cows in mid-lactation when fed by mouth. Further preparations made from Ardein were of low potency as judged by their chemical analyses. In an experiment with dairy cows the most promising of a whole series of Ardein preparations (iodinated Ardein N9 + 10\*) failed to stimulate milk production or metabolism to any appreciable extent. In doses of 50 g., these iodinated Ardein preparations produced unmistakable symptoms of iodine poisoning in one of the experimental cows [Blaxter, 1943a]. Iodinated casein preparations, however, appeared more promising from the chemical data, and experiments have since been carried out using some of the preparations described in Pitt Rivers & Randall's paper.

\* The nomenclature of these preparations is that given by Pitt Rivers & Randall [1945].

The first part of this paper deals with the experiments which were carried out using three preparations of iodinated casein, NC1, NC2 and NC3. Cows in the herd of the National Institute for Research in Dairying were used as experimental animals, and the programme of experiments occupied the summer of 1943. The objects of the experiments were to determine whether these iodinated casein preparations would stimulate milk production, and to elucidate some of the factors concerned in determining the optimal stimulation of the cow.

The second part of this paper is concerned with the incorporation of iodinated casein into dairy cow feeding cubes, these experiments having been carried out with the kind co-operation of Mr J. Hunter-Smith of the Hertfordshire Farm Institute; Prof. R. Rae of the University Farm, Sonning; Mr Henry Robinson of Iford; and Messrs J. and H. Robinson of Deptford Bridge Mills, London.

#### EXPERIMENTS WITH IODINATED CASEINS NC1, NC2 AND NC3

##### *A comparison of the thyroidal activity of the three preparations*

The iodine analyses of the three iodinated caseins were all very satisfactory, for all contained considerably more acid-insoluble ('thyroxine') iodine than iodinated Ardein N4MB. For this reason, it was thought desirable to reduce the dose to 30 g. per day, especially as the experiments with 50 g. of iodinated Ardein N4MB had produced such an obvious hypermetabolism. Table 1 shows the iodine analyses [Pitt Rivers & Randall, 1945] and thyroidal activity as estimated from tadpole assays [Deanesly & Parkes, 1945] of the three preparations. Similar data for the iodinated Ardeins N4MB and N9+10MB are also shown in the table.

Table 1. *Iodine analyses and iodine dosages*

	% iodine			Dosage		
	Total	Acid-insoluble ('thyroxine')	Thyroidal activity*	g.	Total iodine	Acid-insoluble iodine
					g.	g.
Iodinated Ardein N4MB	5.76	0.88	—	50	2.88	0.44
Iodinated Ardein N9+10MB	5.50	0.31	—	50	2.75	0.16
Iodinated casein NC1	6.3	1.0	Poor	30	1.89	0.30
Iodinated casein NC2	7.3	1.2	Good	30	2.19	0.36
Iodinated casein NC3	9.0	2.7	Good	30	2.70	0.84

\* Quoted from Deanesly & Parkes [1945].

With a dose of 30 g. of the iodinated caseins, the iodine intake per day was high, but in view of previous results no symptoms, or, at the most, very slight symptoms of iodine poisoning were thought likely to occur. The iodine analyses and iodine dosages indicated that good responses should be obtained from all three iodinated caseins.

Eight dairy cows were used as experimental animals, and details of these are shown in Table 2.

It will be noted that first calf heifers were included in the experiment as well as mature cows, for there was some reason to suppose that growth might affect the ability of heifers to respond to treatment. The mature cows were an irregular group

Table 2. *Details of experimental animals*

No. of cow	Treatment given	No. of calvings	Date of previous calving	Date of service	Days in milk	Mean daily yield before exp. commenced
A1	I.C. NC1	1	29 Aug. 1942	28 Nov. 1942	270	21.4
A2	I.C. NC2	1	11 Sept. 1942	25 Dec. 1942	257	19.4
A3	I.C. NC3	1	30 Aug. 1942	22 Dec. 1942	269	23.8
AC	Control	1	18 Aug. 1942	26 Nov. 1942	281	17.3
B1	I.C. NC1	3	13 Jan. 1942	Not served	408	17.4
B2	I.C. NC2	2	6 Apr. 1942	15 Jan. 1943	415	17.5
B3	I.C. NC3	5	29 Sept. 1942	18 Nov. 1942	239	15.1
BC	Control	4	21 Sept. 1942	13 Dec. 1942	247	21.0

and were very late in lactation. Four treatments were allocated at random within both the groups or 'blocks', as follows:\*

- '1': supplement of 30 g. of iodinated casein NC1,
- '2': supplement of 30 g. of iodinated casein NC2,
- '3': supplement of 30 g. of iodinated casein NC3,
- 'C': control with no additional supplement.

The experiment was divided into three periods: an initial control period of 10 days; an experimental period of 21 days; and a final control period of 21 days. During the whole experiment the cows were at grass, this feeding being supplemented with a mixture of concentrated foods in which the iodinated casein was mixed. The pasture deteriorated as the experiment progressed and was changed during the final control period. The concentrate mixture was

Ground oats and barley	1 part
Sugar-beet pulp (dried)	1½ parts
Bran	1 part
Decorticated ground-nut cake	½ part
Decorticated ground-nut meal	½ part

It was fed according to milk production, allowance being made for the variation in the feeding value of the pasture from week to week.

### Results

The individual doses of iodinated casein were weighed daily and carefully mixed with each cow's morning and evening meal of concentrates. At the first meal there were no refusals, although cows B2 and B3 tended to scatter their food considerably. No refusals occurred on the next day, but on the third day cows A3 and B3 refused food, while cow B2 commenced refusing food on the fourth day. Every endeavour was made to ensure a complete consumption of the ration by the use of condiments, and eventually it was found that freshly ground linseed cake was the most effective supplement. Subsequently all cows, including the controls, were given 25 g. of linseed cake meal daily. No refusals of food occurred following the fifth day, with the exception of cow B2, which refused on several occasions despite every artifice used for her persuasion. After 7 days of linseed cake meal admixture it was found that small quantities of iodinated casein were sifting through

\* These iodinated caseins were 'coated' with stearic acid, but no additional fatty acid or protein was given to the control cows, as it had been found that such supplements exerted no effect whatever.

the mixture of concentrated foods and were not being consumed. For this reason 25 ml. of a dilute solution of molasses was mixed with each cow's concentrate ration before the admixture of the iodinated casein. This effectively prevented sifting and a total consumption was the result.

In the summer months cows often fail to consume the whole of their supplementary ration, especially when the grass supply is good and when supplementary feeding is in a meal rather than a 'cube' form. For this reason a comparison of the palatability of the iodinated caseins with the palatability of iodinated Ardein is not justified. As neither of the control cows refused food, neither of the cows which received iodinated casein NC1 refused, one cow which received iodinated casein NC2 refused, and as both cows which were fed iodinated casein NC3 refused their ration, the palatability of the three preparations was  $NC1 \gg NC2 > NC3$ . Palatability thus seems to be inversely proportional to iodine content. Only in the case of cow B2 were the refusals of food sufficiently large and prolonged to vitiate the results.

The milk produced by each cow was weighed and recorded at every milking. These data are given in Table 3.

Table 3. *Mean daily milk yields in lb.*

No. of cow	Control period	Experimental period			Final period		
		Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
A1	21.1	18.7	17.6	15.9	13.4	10.9	9.9
A2	19.4	19.4	22.4	22.4	18.0	14.0	11.0
A3	23.0	21.0	27.6	27.0	21.3	14.7	15.3
AC	17.5	17.2	17.0	16.1	15.6	14.8	15.2
B1	18.1	18.3	18.3	16.9	12.2	11.6	10.9
B2	16.3	12.4	8.1	3.8	1.7	0.0	0.0
B3	13.8	12.5	10.3	8.7	6.6	3.2	0.0
BC	20.7	17.6*	12.4	8.0	Removed from experiment		

\* Onset of lameness.

The interpretation of the above table is complicated, owing to the fact that the two groups or 'blocks' were not comparable. In the heifer block, the data indicate a considerable increase in milk production in the case of those that received iodinated caseins NC2 and NC3, but no change was evident in the production of the heifer which received iodinated casein NC1. Owing to the differences in the normal rates of decline in milk yield of these animals, responses have been calculated as deviations from a regression line fitted to the daily milk yields in the initial period and the last week of the final period. The mean daily changes in milk yield ('actual yield minus expected yield') gave the following results during the last 2 weeks of experimental feeding.

Heifer A1 (iodinated casein NC1)	+0.4 lb. per day
Heifer A2 (iodinated casein NC2)	+6.5 lb. per day
Heifer A3 (iodinated casein NC3)	+7.5 lb. per day
Heifer AC (control)	-0.3 lb. per day

It can thus be concluded that both iodinated caseins NC2 and NC3 gave good responses, but iodinated casein NC1 was without substantial effect on milk production.

In the cow block, however, the result was not so clear-cut. The control cow developed acute lameness and had to be removed from the experiment as her treatment had become abnormal, but, even so, the two cows which received iodinated caseins NC2 and NC3 failed to respond in the way that the comparable heifers had done. Both declined in yield rapidly and were dry at the end of the experiment. The cow which received iodinated casein NC1 maintained her production but showed no response in yield, confirming that NC1 was not potent. In the case of cow B2 the failure to consume the ration was probably the reason for the absence of a response, but cow B3 only refused food on three occasions, the same number of occasions as the comparable heifer A3. It appears probable that in the final stages of lactation it is not possible to elicit a response. This can only be a correct conclusion if the metabolism of the cow was demonstrably increased by iodinated casein treatment.

As in the case of previous experiments, heart rates, respiratory rates, and rectal temperatures were recorded to determine whether any changes in the metabolism of the animals had occurred. Heart rates were taken between 6.30 and 7 a.m. and again between 4 and 4.30 p.m. Respiration rates and body temperatures were taken once daily during the morning. The data for each cow are shown in Table 4. It was apparent that the metabolism of three of the cows had increased considerably, for the heart rates of cows A2, A3 and B3 were considerably elevated. Respiration rates were elevated slightly in the case of cows A3 and B3, while there was an increase in the body temperature of cows A2, A3, B2 and B3. Thus iodinated casein NC1 failed to increase the metabolism of the two cows A1 and B1, in the same way that it failed to increase their milk production. Iodinated casein NC2 increased the metabolism of cow A2, but only a slight increase of body temperature was seen in cow B2, the cow which failed to consume her ration and failed to increase in milk production. While cow A3 increased in milk yield and in metabolism, the metabolism of cow B3 was elevated but no response in production occurred, showing that although it is possible to increase the metabolism of cows when their lactation has reached the phase of rapid terminal decline, such iodinated protein stimulation is without a demonstrable effect on milk production.

The cows were weighed on three consecutive mornings at 3-weekly intervals, once before treatment commenced, once during the last week of experimental treatment, and again during the last week of the final control period. These data are also shown in Table 4. Unfortunately, data are not available for the control cow BC, but nevertheless the three cows for which elevation of the heart rate was noted lost weight considerably during the experimental period. The loss of weight was only reflected in a loss of condition in cow A3, and a slight gain in condition could be observed in the case of cows A1, B2 and AC. There was no observable change in the condition of cows B1, A2, or B3. Following the cessation of treatment all cows gained rapidly in weight, part of the gain in weight in the case of cows B2 and B3 undoubtedly being due to pregnancy, but those cows which had lost weight tended to gain more than the others in the subsequent period.

On each day of the experiment each cow was inspected and any abnormality recorded. Three of the cows had slight eye discharges during the experimental feeding. Cow A3 discharged on the 3rd, 9th, 10th, 11th, 16th and 20th days but not in either of the control periods. Cow A2 discharged on the 2nd, 11th and 20th days of treatment

Table 4. *Mean heart and respiratory rates per minute and rectal temperatures in °F.*

Cow no.	Initial control period	Experimental period			Final period		
		Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
		Heart rate					
A1	80.7	77.8	79.4	78.1	79.5	76.1	79.3
A2	71.1	74.0	79.0	83.0	74.5	64.2	64.0
A3	76.8	82.7	87.2	91.7	82.6	66.8	65.2
AC	77.4	73.3	74.6	75.9	76.7	71.1	72.0
B1	60.8	60.7	60.9	53.3	60.1	56.6	57.0
B2	63.9	63.4	64.3	62.5	67.1	62.6	63.7
B3	70.2	71.3	79.2	81.6	80.7	64.8	63.2
BC	65.3	70.5	62.1	58.0	*	*	*
Respiratory rate							
A1	32.2	31.8	31.8	35.0	35.0	32.2	40.0
A2	32.8	31.2	32.8	32.0	31.3	31.2	32.5
A3	37.7	38.1	42.8	42.5	42.3	36.3	34.2
AC	29.3	30.2	29.0	28.7	31.0	28.3	30.5
B1	30.0	31.8	29.7	32.8	38.5	33.3	33.3
B2	32.5	30.8	29.8	33.0	32.7	30.0	35.0
B3	34.2	31.7	34.7	36.3	38.7	32.0	34.0
BC	34.1	37.5	29.3	32.3	*	*	*
Rectal temperature							
A1	101.69	101.53	101.64	101.70	101.68	101.93	102.11
A2	100.69	100.83	101.06	101.15	100.86	100.79	101.13
A3	101.31	101.30	101.43	101.73	101.55	101.38	100.88
AC	100.80	100.66	100.55	100.70	100.85	100.96	100.96
B1	100.53	100.66	100.55	100.55	101.06	100.94	101.08
B2	101.12	101.01	101.31	101.35	101.58	101.53	101.36
B3	101.12	101.05	101.21	101.45	101.48	101.10	101.21
BC	101.18	101.26	101.00	101.20	*	*	*
Change in weeks 2 and 3 from initial period				Change in live weight in lb. during period			
Cow no.				Rectal temp. °F.	Initial period to week 3 of the experimental period		Week 3 of the experimental period to week 3 of the final period
	Heart (beats)	Respiration (no.)					
A1	- 1.95	+1.20	-0.02	+40.0	+ 44.0		
A2	+ 9.90	-0.80	+0.41	-15.0	+ 62.3		
A3	+12.65	+4.95	+0.27	-86.8	+ 79.5		
AC	- 2.15	-0.45	-0.17	+37.7	+ 29.7		
B1	- 1.20	-0.55	+0.02	+21.3	+ 42.0		
B2	- 0.50	-1.10	+0.21	+41.7	+ 77.7		
B3	+10.20	+1.30	+0.21	-23.2	+104.5		
BC	Not calculated			-	*	*	

\* Cow BC became lame and was removed from the experiment.

and again during the final control period. A watery nasal discharge from the nostrils and a clear eye discharge were noted for cow B2 on the 10th day of treatment and again on the 5th day of the final control period. These symptoms were never serious at any time, and in view of the very bright sunshine and the fact that other non-experimental cows were seen to discharge in a similar manner, they cannot be interpreted as iodism symptoms, and most of the lachrymation and conjunctivitis was probably due to the glare of the sun.



Only one heart abnormality was noted, cow A2's heart missing one beat on the 20th day of treatment, but this was not observed again. Cow A1's heart sounds were slightly abnormal (auricular flutter) throughout the whole experiment. The cowman milking the experimental cows made an interesting observation. He found that both cows A2 and A3 milked more easily following treatment, for these cows 'let down their milk' more quickly than formerly was the case. No comparable change was found in the case of cows B2 and B3.

The results of the experiment are interesting in several respects. First, taking into consideration all the factors which were studied it is apparent that iodinated casein NC1 was not potent while both NC2 and NC3 were highly active, iodinated casein NC3 probably being a more highly active preparation than NC2. When these results are related to the acid-insoluble ('thyroxine') iodine dosages which were used agreement is poor, for iodinated caseins NC1 and NC2 should have given approximately equivalent results, while NC3 should have been definitely superior. A possible explanation of the inactivity of NC1 may be that this sample is the only one of which the preparation did not include incubation at a high temperature. The agreement of the cow responses with the tadpole assays of the thyroidal activity of these preparations [Deanesly & Parkes, 1945], however, is considerably better, although neither can be stated quantitatively. Secondly, it appears that heifers in their first lactation respond to thyroxine in a normal way, indicating that there is no growth antagonism to stimulation in animals which are not fully mature. Lastly, increasing the metabolism of cows when late in lactation and when the mammary gland is involuting results in no increased secretion, and it is therefore evident that lactation cannot be prolonged by thyroid stimulation.

*The effect of indoor and outdoor feeding conditions and the effect of stearic acid coating on the response to 30 g. of iodinated casein NC3*

The potency of the three preparations thus established, further experiments were carried out, using the most potent preparation, iodinated casein NC3. All the experiments in which a successful stimulation of milk production had occurred had been conducted during the autumn and winter period, with the exception of the last experiment. It was noted, too, that the average heart rates were lower in the initial periods of the experiments which had been conducted in the winter time. It appeared, therefore, that increases in production even greater than the increases obtained in the previous experiment might occur during the winter time, when the cow's metabolism tends to be lower. It was also important to verify whether stearic acid coating of iodinated casein [Blaxter, 1945] had any appreciable effect on its potency. For these reasons an experiment was designed to find whether a reduction in the metabolism of the cow would occur when winter rations were fed in the summer time, whether under such conditions there would be an increased response in milk production, and whether coating of preparations with stearic acid by the method previously described would increase the potency of an iodinated casein.

*Plan of experiment*

Twenty cows were used as experimental animals, and details of them are given in Table 1 of the Appendix. They were grouped according to breed, age and stage of

lactation into four 'blocks' each of five animals. The first block consisted of five second-calf shorthorns, the second of five shorthorn or shorthorn-type cows in their first lactation, the third of more mature shorthorns, while the last block consisted of Guernseys. The five cows in each block were then allocated by the throw of a die to one of the five following treatments.

'A', 'Control out', consisting of pasture feeding conditions throughout the experiment.

'B', 'Coated out', consisting of exactly the same conditions of feeding and management as the cows in group A, but with the daily addition of 30 g. of iodinated casein NC3 coated with 30 g. of stearic acid.

'C', 'Uncoated out', consisting of exactly the same conditions of feeding and management as group A, with the addition of 30 g. of uncoated iodinated casein NC3.

'D', 'Control in', consisting of winter feeding, the cows being allowed access to a bare pasture for 2 hr. each day for exercise purposes.

'E', 'Coated in', consisting of the same feeding and management as the cows in group D, but with the addition of 30 g. of iodinated casein NC3 coated with 30 g. of stearic acid.

The experiment was divided into four periods. During the first 3-week period, all cows were kept at pasture; in the second, which was of 1 week's duration, groups A, B and C remained at pasture, while groups D and E reverted to indoor feeding, these first two periods comprising the initial control period. Experimental treatment commenced in the third period, on 25 June, and the iodinated casein was fed for 3 weeks. Iodinated casein feeding was then stopped and in the last period, which was of 3 weeks' duration, the basal rations of the groups remained unaltered, that is, groups D and E received winter rations and were confined in the cowshed for most of the 24 hr., while groups A, B and C continued to receive their normal summer rations.

The rationing of the three groups at pasture was similar to that carried out in the previous experiment, the same concentrate mixture being used. The cows in groups D and E were fed a ration consisting of seeds hay to cover their maintenance requirements, and concentrated food was fed in addition according to the amount of milk produced.

### Results

The plan of the experiment makes it possible to make a number of comparisons between treatment effects. Data were collected throughout the 10-week period on the milk production, milk composition, and measurements of the total metabolism of each cow, together with daily notes on any abnormality which was observed. The considerable numerical data are not given in full, but summaries are given in the Appendix, while the calculated results with an indication of their statistical validity are given in the text.

There was a plentiful supply of grass during the initial control periods, and the cows were not eager to consume their small ration of concentrates. For this reason all cows were given 30 ml. of a dilute solution of molasses during both the initial periods and the experimental period. This was not given during the final period. The iodinated casein was fed twice daily and was mixed with 50 g. of linseed cake

meal before it was incorporated in the diet. Both groups of control cows received linseed cake meal, but stearic acid or pure casein was not given, for it had been found that such small quantities of additional protein or fatty acid had no effect on either milk production or metabolism.

In spite of these elaborate precautions, food refusals occurred. These were sporadic in the case of cows 16 and 4, but were prolonged in the case of cow 1. In each case, however, the refusal of the concentrate ration or part of it was sufficient to reduce the intake of iodinated casein very considerably. Cows 16 and 4 were Guernseys, which are recognized as fastidious feeders, while cow 1 was a Shorthorn. All three received coated iodinated casein NC3. The smell of the coated material was unpleasant, for the stearic acid was a commercial sample, and it is probable that this was the main reason for the food refusal, especially as the cows fed uncoated material showed no signs of noticing anything abnormal in their ration. Nevertheless, these refusals must be taken into account in interpreting the results.

*The effect on milk production.* Milk yields were recorded twice daily to the nearest  $\frac{1}{2}$  lb., and the mean daily yields at weekly intervals are shown in Table 2 of the Appendix. For the purposes of statistical analysis the mean yield in the initial control periods was calculated and the mean yield deviations from this figure during the experimental and final control periods were found. These data are shown in Table 5.

Table 5. *Surplus over initial control yield during the experimental period and final control period in lb. per day*

Treatment	Experimental period			Mean of weeks 2 and 3	Final control period		
	Week 1	Week 2	Week 3		Week 1	Week 2	Week 3
Control out (A)	+0.09	-2.39	-3.65	-3.02	-4.84	-6.31	-8.10
Coated out (B)	+1.64	+2.97	+1.77	+2.37	+0.91	-5.66	-8.05
Uncoated out (C)	+2.06	+4.57	+4.40	+4.48	+0.16	-5.96	-5.75
Control in (D)	-0.88	-2.11	-3.02	-2.56	-3.73	-4.83	-6.23
Coated in (E)	+1.38	+3.21	+2.59	+2.90	+0.83	-5.79	-5.59

Statistical analysis was then carried out on the mean changes in yield during the last 2 weeks of experimental treatment. Analysis of the variance was used, and the four degrees of freedom for treatment effects were partitioned in four ways to show the statistical significance of the main factors of importance. The analysis of variance is shown in Table 6.

Table 6. *Analysis of the variance of the changes in milk production*

Component	Degrees of freedom	Mean square	F calculated
Blocks	3	16,528.79	23.9**
Treatment	4	9,084.08	13.1**
(a) Coated v. control†	1	23,050.92	33.3**
(b) Outside v. inside†	1	185.73	3.73 N.S.†
(c) Interaction a x b†	1	10.97	63.09 N.S.†
(d) Coated v. uncoated‡	1	1,749.20	2.51 N.S.
Residual (error)	12	692.14	—
Total	19	—	—

\*\* Significant when  $P < 0.01$ .

† Division of three D.F. for treatments A, B, D and E, effect of treatment C removed.

‡ Error variance the great variance. N.S. Not statistically significant.

§ D.F. for B versus C comparison, effect of treatments A, D and E removed.

It will be noted from the analysis of the variance that although there was a significant difference between treatment effects, this was only due to the difference between the drop in the yield of the control cows, and the increase in yield of those cows fed iodinated casein. The effect of coating on the response and the effect of the basal ration on the response (interaction  $a \times b$  in Table 6) were not statistically significant. The significant differences for testing the means in column 4 of Table 5 were:

At odds of 19 : 1	2.89 lb. per day
At odds of 99 : 1	4.06 lb. per day

The standard error of the difference between any two of the means was  $\pm 1.33$  lb. This standard error is relatively large in view of the number of cows employed, despite the fact that the accuracy of a milk yield experiment tends to be lower during the summer months. On inspection of the data this was seen to be due to an enormous range of variability in the responses ranging from +2.79 to +8.81 lb. per day. This was found to be largely a result of the refusal of food by three of the cows. Responses were therefore calculated from only those cows which consumed their ration, and the results are shown in Table 7.

Table 7. *The response to treatments when the results from cows which refused iodinated casein are ignored*

Response to	Response calculated from all cows. Response in lb.	Response calculated from those cows which did not refuse food				
		No. of cows	Response in lb.	Mean daily yield	% response	
					(a)*	(b)*
Coated out (B)	+ 5.39	2	+ 6.52	19.2	33.8	34.5
Uncoated out (C)	+ 7.50	4	+ 7.50	21.7	34.6	36.9
Coated in (E)	+ 5.46	3	+ 6.07	18.7	32.4	33.2
C-B	+ 2.11	—	+ 0.98	—	0.8	2.4
$\frac{C-B}{B}$ %	+39 %	—	+15 %	—	+2 %	+7 %

\*  $a$  = column 4 as a percentage of column 5;  $b$  = mean of individual % responses.

Table 7 shows that when the refusal of food is ignored there was a difference in favour of uncoated iodinated casein of 2.11 lb. per day above coated material when fed under comparable conditions—a 39 % advantage. When only those cows which consumed the whole of their ration were compared, the advantage was reduced to 15 %. There was, however, a slight difference in the initial yield of the cows in the two groups, and when the percentage responses were calculated, there was only an extremely small advantage in favour of non-coating. Statistical analysis of the percentage responses confirms that coating with stearic acid is without effect on the milk yield response to a standard dose of iodinated casein NC3.

In view of the absence of any statistically significant differences between the treatment effects, and because of the possible effect of the initial level of production of the cow on the response which she gives to a standard dose of iodinated casein, the individual increases in yield (corrected for the decline in the yield of the control cows) were calculated and are shown in Table 8. The cows have been arranged in order of milk yield response and the treatment which they received is also shown.

Table 8 also shows the responses in heart rate and in fat production calculated in the same manner, and these responses will be referred to later.

Table 8. *Individual responses in milk yield, fat yield, and heart rate to 30 g. of iodinated casein NC3 during the last 14 days of treatment*

Cow no.	Treatment	Whether refused food	Daily milk yield in lb.			Weekly fat yield in lb.			Heart rate (beats per min.)		
			Mean initial yield in lb.	Response in lb.	% response	Mean initial yield in lb.	Response in lb.	% response	Mean initial rate	Beats increase	% increase
1	B	Refused	19.5	+2.75	+14.1	5.83	+1.28	+21.9	63.4	+10.2	+16.2
16	E	Refused	28.5	+3.51	+12.3	9.37	+2.90	+30.9	68.2	+15.2*	+22.3
3	B	No	13.7	+4.92	+35.5	4.00	+1.30	+32.5	72.0	+30.7*	+42.7
13	E	No	14.1	+5.41	+38.2	3.37	+1.93	+57.3	59.3	+12.1	+20.3
4	B	Refused	23.1	+5.71	+24.7	7.78	+2.25	+28.9	64.0	+10.5	+16.4
5	C	No	17.7	+6.02	+34.3	3.97	+2.43	+61.2	65.2	+17.7*	+27.2
15	E	No	19.5	+6.22	+31.9	4.73	+2.90	+61.3	68.4	+23.0	+33.6
14	E	No	22.3	+6.58	+29.5	5.99	+3.11	+51.9	67.0	+30.1	+36.1
6	C	No	22.1	+6.82	+30.8	5.32	+2.00	+37.6	65.7	+15.4	+23.4
2	B	No	24.5	+8.23	+33.6	5.38	+2.99	+55.6	71.5	+16.5	+23.0
8	C	No	27.3	+8.41	+30.8	8.29	+4.16	+50.2	63.1	+21.8	+33.7
7	C	No	19.7	+8.81	+44.8	5.14	+3.38	+65.8	64.8	+22.2	+35.3

\* See observations on clinical abnormalities.

When plotted, the data on milk production for individual cows show that the response of cows which refused food was below that of those which consumed the whole of their ration. The correlation coefficient  $r$  calculated from the remaining nine cows relating the increase in milk production in lb. per day to the mean initial yield was +0.7874, significantly different from zero correlation at odds of 82:1, and indicating that approximately 60% of the variation in the responses to a standard dose was associated with variation in the initial yield of the cows. The corresponding regression equation was

$$R_{lb.} = 0.241 Y_{day} + 1.89,$$

where  $R_{lb.}$  = response in lb. per day to 30 g. of iodinated casein NC3,

$Y_{day}$  = average initial milk yield of the cow in lb. per day.

This equation indicates that a cow yielding 1 gal. of milk per day will increase in milk production 4.4 lb. a day when 30 g. of iodinated casein are fed, while one yielding 3 gal. would increase in yield 9.2 lb. a day. The fact that there is a positive intercept of 1.89 lb. means that the percentage response must decline with increasing yield, otherwise the intercept would be zero. For this reason the correlation of the percentage response with initial yield was calculated. This was negative and only statistically significant at odds of 8:1 ( $r = -0.4668$ ). Here the regression equation was

$$R_{\%} = 44.30 - 0.4935 Y_{day},$$

where  $R_{\%}$  = the percentage response to 30 g. of iodinated casein NC3.

Care has to be taken in interpreting these results, for the yield of a cow per day prior to treatment is a composite term related to her inherent productivity and her stage of lactation prior to treatment. Thus it cannot be concluded that low-yielding

cows respond more than high-yielding cows, for, in this experiment, the stage of lactation was a more important factor determining the level of initial yield than inherent productivity. The stage of lactation, therefore, appears the main factor involved. When more data are available, the relationship between true productivity and the lactation stage of the cow as factors determining the magnitude of a response can be analysed more fully. This initial analysis, however, confirms for iodinated casein stimulation the observations made by the Missouri workers, who found that the relative increases in milk production to injections of thyroxine on three successive days diminished with increasing productivity [Ralston, Cowser, Ragsdale, Herman & Turner, 1940].

The data on the daily changes in milk yield for the nine cows which did not refuse food were used to form an accurate picture of the time relationships of dosage. These data, plotted in Fig. 1, show that the response is a continuous one, and the

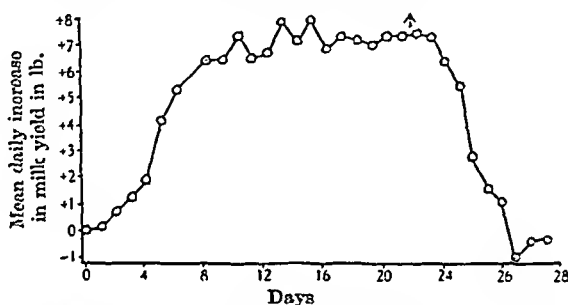


FIG. 1. The time curve of the response in milk yield to continuous feeding with 30 g. of iodinated casein NC3.

time/response curve is sigmoid, not a sudden response occurring on the 5th day, but a gradual one. The rate of response increased until the 6th day and subsequently decreased. The fall in production at the cessation of treatment was much more rapid than the increase in production when treatment commenced.

*The effect on milk composition.* Samples of milk were taken on two consecutive mornings and two consecutive evenings during each week of the experiment, and fat percentages were determined on bulked evening and morning samples for each cow. The mean data are shown in Table 3 of the Appendix. Calculation of the mean change in the fat percentage from the preliminary period to the experimental period was carried out and is shown in Table 9.

Table 9. *Mean change in the fat percentage of the milk during the last 2 weeks of experimental treatment*

Treatment	Units change in fat %
A, Control out	+0.264
B, Coated out	+0.456
C, Uncoated out	+0.595
D, Control in	+0.343
E, Coated in	+0.934

Statistical analysis of the results, and correction of the results for refusals of food showed that while iodinated casein increased the fat percentage, coating the iodinated

casein and differences in the basal ration were without significant effect on the response. The individual responses were, however, very variable, much more so than was the case in the milk production responses. There was some indication that the response was greatest for those cows with an initially low fat percentage, but statistically this was only significant at odds of 11 : 1. This association may well have been a stage of lactation effect, for normally the fat percentage increases during the late stages of lactation. In connexion with the variability of the response in the fat percentage it must also be remembered that the fat percentage was determined on a smaller number of occasions than was milk production, and the accuracy of the determination of the fat percentages is thus very low.

The average increase in the fat percentage of those cows which did not refuse food was 0.366 % units or 8.9 %. As milk production increased considerably, fat production was highly elevated, and the calculated weekly yields of fat are shown in Table 4 of the Appendix, while the mean changes during the last 14 days of treatment are given in Table 10.

Table 10. *Mean change in the weekly yield of fat during the last 2 weeks of experimental treatment*

Treatment	Responses		Mean change in fat yield (lb. per week)		
	Actual response		Response excluding those cows which refused their food		
	lb., per week	%	No. of cows	lb. per week	%
A, Control out				-0.408	
B, Coated out	+1.953	+40.0	2	+2.145	+45.7
C, Uncoated out	+2.990	+52.6	4	+2.990	+52.6
D, Control in	+2.714	+40.3	3	+2.647	+55.1
E, Coated in				+2.390	

Table 10 is similar to Table 7. It shows that fat production was decidedly increased and that when only those cows which consumed the whole of their ration are compared there was a slightly closer agreement between the effects of feeding iodinated casein under the three sets of experimental conditions. Statistical analysis of these data indicates that there was no significant difference between these methods.

There was a decided range of variability in the fat production responses, shown in Table 8. The percentage increases in fat production were all greater than the percentage increases in milk production except in cow 3. This cow was very late in lactation and had a very high fat percentage during the initial period. The correlation ( $r$ ) between the initial fat production and the increase in fat production was +0.7998, which is of the same order as the correlation between initial milk yield and the milk yield response to iodinated casein NC3. The regression equation was

$$R_F = 0.4883 Y_F + 0.209,$$

where  $R_F$  = the response in the weekly fat production to 30g. of iodinated casein NC3,  
 $Y_F$  = the initial fat yield of the cow in lb. per week.

The slight positive intercept indicates that the response is proportionately smaller at higher yields; thus a cow yielding 1 gal. of milk per day with 3.5% fat would produce 1.39 lb. or 57% more fat per week, while one yielding 3 gal. a day of the same quality milk would produce 3.56 lb. or 48% more fat in a similar period. Though this estimate is subject to a considerable error, it appears that 65% of the variation in the increase in fat production is determined by the initial level of fat secretion of the cow, and the relationship confirms that found by Ralston *et al.* [1940].

The total solids of the milk were determined gravimetrically on the same number of occasions as was the fat content, and the percentage of solids-not-fat was determined by difference. These data are shown in Table 5 of the Appendix. Analysis of these data showed that there was little change which could be ascribed to the effect of feeding iodinated casein, and certainly no changes connected with stearic-acid coating of the iodinated casein or with a variation in the basal ration. It is obvious, however, that the solids-not-fat production of the cows increased, for the milk yield of the cows treated with iodinated casein NC3 was substantially elevated. There was, however, a tendency for the solids-not-fat percentage to be depressed when treatment stopped. Taking only those cows which consumed the whole of their ration, and comparing the difference between the solids-not-fat percentage during the initial periods with the solids-not-fat percentage in the last 2 weeks of the final period, the solids-not-fat percentage in the milk had fallen 0.111% units, whereas in the case of the control cows the fall had only been 0.032% units. The difference was, however, not significant statistically.

The phosphatase of the milk was kindly determined by Dr S. J. Folley on composite samples of milk. As in previous experiments with both thyroxine injection and with iodinated protein feeding [Folley & White, 1936; Blaxter, 1945] there was a decline in the milk phosphatase when iodinated casein was fed, and, following the cessation of treatment, a rise occurred to a level comparable with the level characteristic of the initial periods. There was no difference between the three treatments involving iodinated casein feeding. The data are shown in Table 6 of the Appendix.

*Physiological effects.* The heart rates of the cows were taken by direct auscultation as in the previous experiments. They were recorded in the morning between 6.15 and 8.15 a.m. and again in the afternoon between 3.15 and 5 p.m. on 6 days of each week of the experiment. The respiratory rates and rectal temperatures of each cow were determined in the mornings. The mean data are shown in Tables 7, 8 and 9 in the Appendix and the mean changes in the rates and temperatures of the animals are shown in Table 11, together with the calculated responses.

It was apparent that there was a large increase in the heart rates of the cows which were fed iodinated casein NC3 and a decrease in the heart rates of the control cows, the decline being greater for those controls which were at pasture. This difference between the two groups of control cows reflects the differences in nutritional level between the two groups. While the nutritional plane of the cows maintained on winter rations remained constant, the grass supply at pasture decreased considerably owing to very dry, hot weather, and the heart rates of the cows in group A were not maintained at the high levels characteristic of the control period. These results are shown in Fig. 2. As in the case of milk yields and fat yields, the data were analysed statistically and no differences between the three iodinated-casein treatments were



Table 11. *Mean changes in heart rate and respiratory rates per minute, and mean changes in rectal temperature in °F. based on values obtained during the initial periods*

Treatment	Experimental period			Final period		
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
<i>Heart rate (beats per min.)</i>						
Control out (A)	+ 1.96	- 2.25	- 5.98	- 3.33	- 6.29	- 8.98
Coated out (B)	+ 10.64	+ 13.98	+ 14.08	+ 3.71	- 8.15	- 12.19
Uncoated out (C)	+ 8.27	+ 15.79	+ 16.85	+ 5.58	- 8.33	- 12.23
Control in (D)	- 2.48	- 2.02	- 2.23	- 1.12	- 0.89	- 1.19
Coated in (E)	+ 5.71	+ 14.94	+ 18.10	+ 9.33	- 3.04	- 4.21
<i>Response:</i>						
Coated out	+ 8.68	+ 16.23	+ 20.06	+ 7.04	- 1.86	- 3.21
Uncoated out	+ 6.31	+ 18.04	+ 22.83	+ 8.91	- 2.04	- 3.25
Coated in	+ 8.19	+ 16.96	+ 20.42	+ 10.45	- 2.15	- 3.02
<i>Respiratory rate per min.</i>						
Control out (A)	+ 0.62	- 0.46	+ 0.12	- 1.08	+ 0.63	- 2.33
Coated out (B)	+ 5.64	+ 6.04	+ 8.83	+ 4.08	- 0.04	- 2.41
Uncoated out (C)	+ 2.41	+ 2.87	+ 7.96	+ 0.12	- 0.25	- 2.58
Control in (D)	+ 0.04	- 1.67	+ 1.08	+ 1.04	+ 2.25	+ 0.12
Coated in (E)	- 0.29	- 1.62	+ 7.79	+ 3.37	+ 2.37	+ 1.04
<i>Response:</i>						
Coated out	+ 4.92	+ 6.50	+ 8.71	+ 5.16	- 0.67	0.08
Uncoated out	+ 1.79	+ 3.38	+ 7.85	+ 1.20	- 0.29	- 0.25
Coated in	+ 0.33	- 0.05	+ 6.71	+ 2.33	+ 0.12	+ 0.92
<i>Rectal temperature °F.</i>						
Control out (A)	- 0.07	- 0.01	- 0.05	+ 0.05	+ 0.11	+ 0.17
Coated out (B)	+ 0.19	+ 0.23	+ 0.18	+ 0.21	- 0.04	+ 0.03
Uncoated out (C)	+ 0.03	+ 0.10	+ 0.20	- 0.02	- 0.12	+ 0.05
Control in (D)	+ 0.04	+ 0.17	+ 0.31	+ 0.21	+ 0.19	+ 0.24
Coated in (E)	+ 0.10	+ 0.17	+ 0.42	+ 0.20	+ 0.01	+ 0.07
<i>Response:</i>						
Coated out	+ 0.26	+ 0.24	+ 0.23	+ 0.16	- 0.15	- 0.14
Uncoated out	+ 0.10	+ 0.11	+ 0.25	- 0.07	- 0.26	- 0.12
Coated in	+ 0.06	0.00	+ 0.11	- 0.01	- 0.18	- 0.17

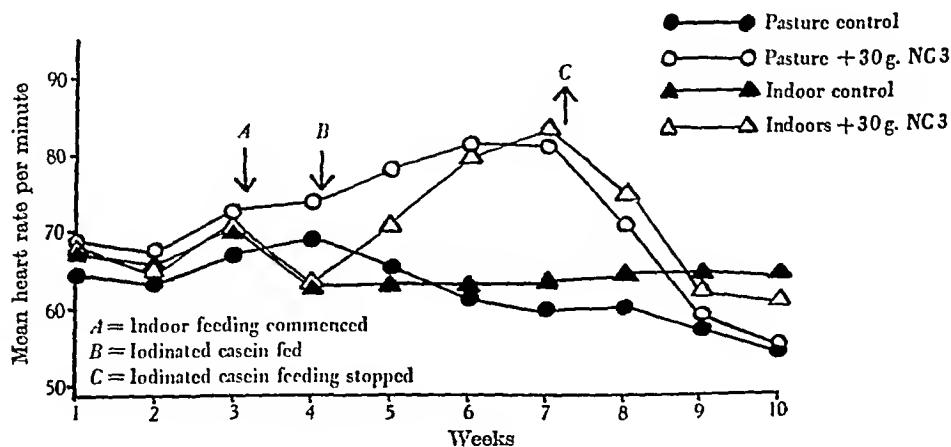


FIG. 2. The effect of differing conditions of feeding on the heart rate and on the increase in the heart rate when 30 g. of iodinated casein NC3 were fed.

Table 12. *Mean heart rates during the last 2 weeks of experimental treatment when the results from cows which refused their food are ignored*

Response to	Response calculated from all cows		Response calculated from those cows which did not refuse food			
	Beats per min.	% response	No. of cows	Response in beats per min.	Initial mean rate (beats per min.)	% response
Coated out (B)	+18.14	+26.8	2	+23.6	+71.7	+32.9
Uncoated out (C)	+20.43	+31.6	4	+20.4	+64.7	+31.6
Coated in (E)	+18.69	+28.4	3	+19.8	+64.9	+30.5

found. The extent to which differences between treatment means were complicated by food refusals is shown by the results in Table 12.

Correction for refusal of food shows that there was little difference between the three iodinated casein treatments, and this was confirmed by simple statistical tests. There was, however, a considerable variation in the responses in the heart rate of individual cows as shown in Table 8. The variation was greater than the variation in milk production, for the coefficient of variation in the heart rates was 30.2%, while for milk production it was 19.1%. There was a very broad agreement between the heart rate responses and the milk yield responses both calculated as percentages but in view of the effect of initial milk yield on the milk yield response and of the possible existence of other factors, an analysis has not been carried out to determine the approximate nature of this relationship. Ralston *et al.* [1940] found that those cows with the highest initial heart rates showed the smallest increase in heart rate when a standard injection of Na-thyroxine was given during a 3-day period. They have interpreted this as an indication that a low initial level of metabolism is important in securing the greatest rise in the metabolism per mg. of thyroxine. Data on the heart rate responses of individual cows which responded in milk production by between 30 and 35% were abstracted from the results of the experiments with iodinated casein and those with iodinated Ardein and Na-thyroxine [Blaxter, 1945]. It was found that the heart rates of cows with low initial heart rates (*circa* 50) increased by 19.5 or 38.9%, while those with high initial heart rates (*circa* 70) increased by 13.9 beats or 20.4%. This is in agreement with the results obtained by Ralston *et al.*, but in view of the fact that the increase in milk production was between 30 and 35% in each case, a more logical explanation is that the relationship between the heart rate and metabolism is not one of direct proportionality and the curve relating the heart rate to total metabolism is concave downwards. At the cessation of treatment there was a fall in the heart rates of the treated cows below the level of the control cows, and in the third week of the final period the heart rates of the iodinated-casein-treated cows were 3.0–3.5 beats below the controls. This was associated with a slight depression of the rectal temperature, and although not significant statistically may indicate that the post-experimental depression of milk production is a result of a lowering of the total metabolism.

There was an increase in the respiratory rates of the cows during the period of iodinated-casein stimulation, and statistical analysis shows that while this was highly significant no differences between the three iodinated-casein treatments were

apparent. Rectal temperatures also increased, but the increase was small and did not represent a serious hyperthermia.

The body weights of the cows were determined on three consecutive days before treatment, at the end of treatment, and at the end of the final period. The gains and losses of body weight are shown in Table 13.

Table 13. *Mean changes in body weight in lb.*

Treatment	Mean initial weight	Loss or gain in 21 days of treatment		Loss or gain in 21 days following cessation of treatment	
		Actual	Net	Actual	Net
Control out (A)	1134	+41.2	—	-20.9	—
Coated out (B)	1181	-26.7	-67.8	+27.5	+48.4
Uncoated out (C)	1143	-35.3	-76.4	+24.7	+45.6
Control in (D)	1166	+41.8	—	+2.6	—
Coated in (E)	1182	-52.4	-94.2	+71.1	+68.5

The actual weight losses were not severe except in the case of group E where a loss of body condition was also apparent. The net losses, however, were large, and a comparison of the condition of the cows which received iodinated casein with the control cows showed large differences. The loss of weight was chiefly over the ribs and along the back. There was no wastage of the large thigh muscles [Blaxter, 1945]. Statistical analysis showed that while this net loss of weight was clearly the result of treatment, the difference between the net losses by cows in groups B and E was only significant at odds of 3 : 1. The fact that the cows at pasture lost less body weight was probably related to the observation that the treated cows at pasture tended to graze more heavily (especially cow 8) whereas the indoor cows were strictly rationed. The recovery of body weight was quite marked, a 70 % recovery having occurred by the end of the experiment. The gain in weight of the treated animals was greater for the strictly rationed cows indoors than for those at pasture, probably due to the fall in the grass supply at the time. This poor food supply was shown by the loss of body weight of the control cows at pasture during the final period. There was some relation between the increased metabolism as judged by the heart rates and the loss in body weight. The correlation  $r$  was 0.6510, significantly different from zero at odds of 16 : 1. There was no demonstrable relationship between the loss in body weight and the increase in milk production.

Each cow was examined twice daily throughout the experiment. Severe iodism did not occur although white nasal mucus was exuded on two occasions, and six of the twelve treated cows showed symptoms which could be confused with iodism. It appears, therefore, that an ingestion of 2.70 g. of iodine per day is approximately at the threshold for the manifestation of iodism. Heart rates of over 100 per minute are sometimes reached by perfectly normal cows as the result of activity [Blaxter, 1943b] but resting heart rates rarely approach these levels. Cow 3's heart rate was 125 per minute on the 15th day of treatment and remained high for 2-3 days. Her respiratory rate was between 50 and 70 during this period, and it is obvious that iodinated-casein treatment imposed a considerable strain on this cow. No heart abnormalities were heard, but at the cessation of treatment the rates were extremely variable from minute to minute. Sweating occurred in cows 13 and 15,

both in the indoor group, this probably being a result of the warm air and the lack of air movement in the cowshed at night.

Both treated and control cows lay down in the cowshed during the milking interval, but the treated cows did so on a greater number of occasions. The irritability of the cows increased considerably and this was aggravated by the increase in the number of biting stable flies (*Stomoxys calcitrans*) during the experimental period. The nervousness of the cows was sufficient to make milking difficult. Cow 3 was the most nervous of all the treated cows, and on one occasion when approached unawares she jumped suddenly, slipped, fell down and looked very frightened. The ease of milking increased, especially in cows 7 and 8, and an appreciable swelling of the mammary gland was apparent 8-12 days after treatment commenced. When treatment stopped, the treated cows became more irritable during the 3-4 days in which the rapid fall in yield occurred. This was especially noticeable in cow 8, where on the afternoon of the 4th day following the cessation of treatment the symptoms approached those of a hypoglycaemic fit in the human. An exophthalmos with an increased palpebral fissure was seen in cow 2 but all other abnormalities were not of treatment origin. Cow 14 had a swollen hock with a concomitant rise in body temperature and heart rate, but this was promptly dealt with and she rapidly returned to normal. Cow 16 came into season four times during the experiment, was served on each occasion and has since proved barren.

### Discussion

It is obvious from the above results that 30 g. of stearic-acid-coated iodinated casein NC3 elicited no greater response in either milk production or metabolism than did 30 g. of the uncoated material, and it is therefore apparent that the method used was without any effect on the potency of the preparation. The second comparison which the experiment was designed to examine, however, is more complicated owing to the fact that the total metabolism and milk production were maintained better by the winter ration than by the pasture. This was due to the falling off in the feeding value of the pasture owing to absence of rain. The comparison of responses at two widely divergent levels of metabolism is therefore impossible, but the results show that approximately equal responses can be obtained when widely differing rations are fed.

As there was no differential effect of any of the iodinated casein preparations, the data can be used to find the accuracy of the determination of responses in milk production and metabolism. The data for the nine cows that did not refuse food have been used for this investigation and the results are shown in Table 14.

The most accurate response estimates in this experiment were those related to milk production, while the least accurate was the increase in body temperature. To a certain extent purely statistical errors account for this variation, for body temperatures were only determined six times per week for each cow, and fat percentages only twice per week, but part of the variation is undoubtedly due to a variation between cows in the response they give to a standardized dose. The reasons for such individual variation is apparent in some cases. The individual variation in the normal change of the respiration with a changing environmental temperature is very large, and it has previously been shown that the initial level of production is

Table 14. *Coefficients of variation ( $\sigma_R/M_R \times 100$ ) of estimated responses to 30 g. of iodinated casein NC3*

Factor studied	Mean response	Coefficient of variation %
Increase in rectal temperature	0.244°F.	98.0
% increase in fat %	8.0	97.0
Increase in respiration rate	8.7 per min.	89.1
Units increase in fat %	0.366	88.8
Not loss of weight	83.7 lb. in 21 days	35.9
Increase in fat yield	2.69 lb. per week	32.2
Increase in heart rate	21.1 beats per min.	30.2
% increase in fat yield	52.6	25.8
% increase in heart rate	30.5	24.4
Increase in milk yield	6.82 lb. per day	19.1
% increase in milk yield	34.4	13.0

a factor of importance in determining both the milk and fat production responses. It is evident, however, that from the view-point of the estimation of the activity of a preparation to the dairy cow, more attention should be paid to changes in milk production and heart rate than to changes in less accurate measurements.

The fall in the milk yield response in pounds and the increase in the percentage response in milk production with declining lactation has been confirmed by an experiment with one cow, 'Flora 63'. Flora 63 was a heifer in her first lactation, and had been in milk for 8½ months and in calf nearly 5 months when treatment began. She was given the following treatment sequence.

Period 1: control period of 21 days.

Period 2: feeding period of 21 days.

Period 3: recovery period of 21 days.

Period 4: feeding period of 21 days.

Period 5: recovery period of 21 days.

The whole experiment lasted, therefore, for 105 days, and as there was no comparable animal available for use as a control, the results in the preliminary period and the last week of each recovery period were used to calculate 'expected yields'. 30 g. of iodinated casein NC3 were used during the feeding periods, and an increase in her concentrate allowance was also given at these times. The difficulty of assessment of the nutrients she consumed at pasture means that the results cannot be regarded as an indication of the effect of an increased level of feeding during the treatment periods.

Milk yields, heart rates, respiratory rates, rectal temperatures and body weights were recorded, but the analysis of the results is limited to milk production, heart rates, and body weights, for the inaccuracies inherent in the determination of changes in respiration rate and body temperature make such responses of little value. The heart rate and milk yield of this cow are shown in Fig. 3 and Table 15.

The increases in yield due to treatment were 8.2 lb. or 43 % in period 2 and 5.4 lb. or 66 % in period 4. Using the standard deviation of a milk yield response as found in the previous experiment, this change can be judged significant when  $P < 0.05$ . There was a decline from 25.6 to 20.7 % in the heart rate response during successive dosage periods, but as the standard deviation of a heart-rate percentage is  $\pm 7.4$  %, the

it cannot be concluded that a heart-rate depression occurred during the second period of stimulation. The weight losses, though comparable in the two periods, do not reflect the loss in condition which occurred, for normally the condition and body weights of cows increase very considerably in the late stages of pregnancy. The cow

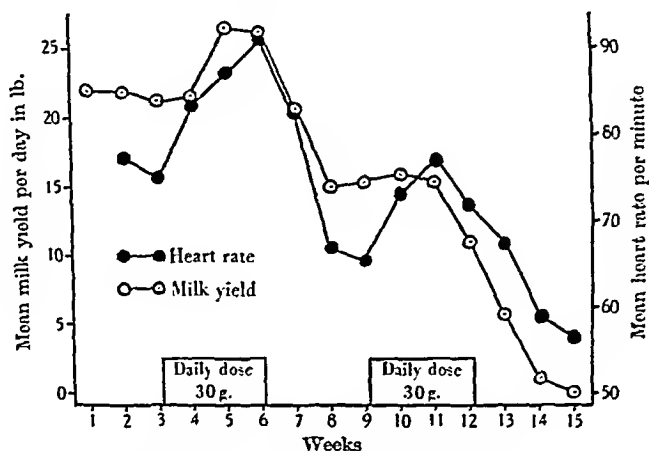


FIG. 3. The effect of two periods of iodinated casein feeding on the milk yield and heart rate of cow Flora 63.

Table 15. *Effect of various treatments on Flora 63*

Period	Week	Mean daily milk yield		Mean heart rate per min.		Mean body weight lb.
		Actual	Expected	Actual	Expected	
1	1	23.7	—	—	—	—
	2	23.8	—	77.7	—	—
	3	22.3	—	74.9	—	1070.3
2	1	22.8	21.0	83.6	73.7	—
	2	27.6	19.7	87.2	72.1	(-70.3)*
	3	27.0	18.4	91.7	70.3	1000.0
3	1	21.2	—	82.7	—	—
	2	14.8	—	66.8	—	(+63.0)*
	3	15.3	—	65.2	—	1063.0
4	1	16.5	11.7	72.9	63.9	—
	2	15.4	9.4	77.2	62.5	(-63.0)*
	3	11.7	7.1	72.0	61.1	995.0
5	1	6.9	—	67.2	—	—
	2	2.0	—	59.8	—	(+50.0)*
	3	0.0	—	56.4	—	1045.0

\* Weight changes during successive periods.

was decidedly poor, her ribs were showing and she carried little fat. Subsequently she was heavily fed, regained but little condition, and calved 5 weeks after the experiment finished. Her calf, a bull, was of normal weight and her milk production in the first 10 weeks of lactation was 35.4 lb. per day, compared with 28.1 lb. in her previous lactation. Nevertheless, the data show that stimulation of the metabolism during the late stages of pregnancy is undesirable, for body tissues are not easily

replaced when the cow is in calf, and she is then liable to calve down in a poor condition, which is not conducive to optimal milk production [Blaxter, 1944].

The data collected on this cow do show that there is a decline in the response in milk production to iodinated casein with decline in lactation, and that the percentage response increases as lactation advances, thus confirming the results of the previous experiment.

*The effect of feeding 15 g. of iodinated casein NC3*

A daily dose of 30 g. of iodinated casein NC3, while increasing milk production quite considerably, had an adverse effect on the cows and the hypermetabolism was revealed in all the physiological records which were taken. It was therefore decided to find whether half this dose (15 g.) would increase production and whether hyperthyroidism would be apparent to such an extent at a lower level of dosage. Six cows in mid to late lactation were used as experimental animals, and, after an initial pairing on the basis of age and productivity they were allocated at random to the two following treatments.

(1) *Controls*: consisting of normal management and pasture feeding supplemented with concentrates.

(2) *15 g. dose*, consisting of the same feeding and management as the controls with the daily addition of 15 g. of iodinated casein NC3.

As in previous experiments, treatment was interpolated between two control periods. It began on 25 July and lasted for 14 days.

30 ml. of a dilute molasses solution were added to each cow's concentrate ration to prevent the small quantity of iodinated casein sifting through the mixture, in the case of the treated cows. The concentrate ration was fed at a constant level throughout the 7 weeks of experiment, and was at all times eaten with relish. It was obvious that the cows could not detect the addition of 15 g. of iodinated casein.

Milk yields were recorded twice daily throughout the experiment and are shown together with the physiological data and the calculated responses in Table 16. Milk yields are also shown graphically in Fig. 4.

Iodinated casein at this level did not give an increase in yield but prevented the normal decline taking place, a net response of 3.04 lb. per day or 16.7% occurring. This net response was only significant at odds of 9:1. There was an increase in the heart rate, however, of 5.72 beats per minute, statistically significant at odds of 29:1. Little change was apparent in the respiratory rate, and although a very slight increase in rectal temperature occurred, this was not significant statistically. The control cows lost 11.4 lb. of body weight during the period, and the treated cows lost 59.9 lb. The difference, or net loss in weight, was 48.5 lb. significant at odds of 18:1.

Following the cessation of treatment the control cows gained 10.1 lb. of body weight, but the treated cows only gained 6.0 lb. This difference was not significant and was not according to expectation, for in previous experiments very rapid gains in weight occurred.

While only the heart rate changes were statistically significant, it is apparent that a slight though distinct effect of feeding 15 g. iodinated casein was evident. A comparison can therefore be made with the results of the previous experiment,

Table 16. *The effect of 15 g. of iodinated casein NC3*

Factor measured	Treatment	Control period		Experimental period		Final period		
		1	2	1	2	1	2	3
Milk yield per day in lb.	Control	19.7	18.4	16.5	15.3	14.9	14.2	14.1
	Treated	18.7	17.6	17.4	17.4	14.6	12.3	11.7
Heart rate: beats per min.	Control	60.6	59.5	57.0	53.9	54.1	54.1	54.5
	Treated	64.6	63.8	65.6	63.7	55.7	56.3	56.7
Respirations per min.	Control	26.3	28.3	30.2	24.5	27.1	30.8	25.3
	Treated	32.1	31.1	36.6	29.9	28.9	30.8	26.3
Rectal temp. °F.	Control	100.8	100.7	100.7	100.8	100.7	101.1	101.1
	Treated	101.1	101.1	100.9	101.2	101.1	101.3	101.4

## Responses

Milk yield in lb. per day	+1.82	+3.04	+0.68	-1.02	-1.48
Heart rate per min.	+4.55	+5.72	-2.11	-1.82	-1.94
Respiration rate per min.	+1.77	-1.72	-2.89	-4.70	-5.72
Rectal temperature in °F.	-0.09	+0.12	+0.01	-0.19	+0.01

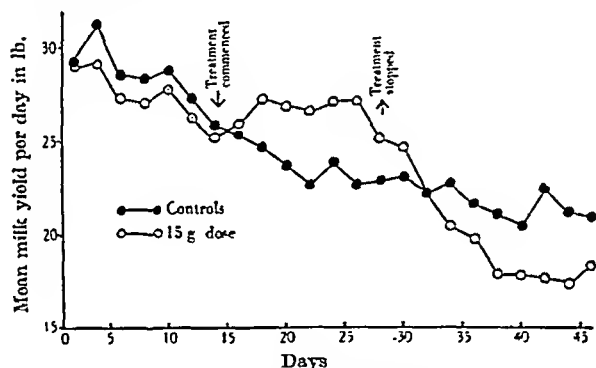


FIG. 4. The effect of feeding 15 g. of iodinated casein NC3 on milk yield.

when 30 g. of iodinated casein was fed, using the responses found during the 7th to 14th days in each experiment for this purpose. This is shown in Table 17.

Table 17. *Mean responses to 15 g. and to 30 g. of iodinated casein NC3*

Factor measured	15 g.	30 g.
Milk yield per day	+ 3.04 lb.	+ 6.57 lb.
Milk yield percentage	+ 16.7	+ 32.7
Heart rate per min.	+ 5.7 beats	+ 19.3 beats
Heart rate percentage	+ 9.3%	+ 25.8%
Respiration rate per min.	- 1.72	+ 3.27
Rectal temperature	+ 0.12	+ 0.11
Actual weight loss	59.9 lb.	14.5 lb.
Loss of gain in weight of control cows	- 11.4 lb.	+ 21.8 lb.
Net loss of weight	48.5 lb.	36.3 lb.
Mean initial yield	18.2 lb.	20.1 lb.

The data in Table 17 referring to the effect of a dose of 30 g. of iodinated casein exclude those cows which refused their food.



It appears that doubling the dose of iodinated casein NC3 doubles the response in milk production. These results indicate that the milk yield response to iodinated casein is probably directly proportional to dosage within this range. The heart-rate responses, however, suggest that the higher dose had a more pronounced effect on the total metabolism of the cow than would be expected on the basis of a directly proportionate relationship. This does not necessarily mean that the increase in metabolism is not directly proportional to dosage for, at the lower dose level, an increase in the metabolism of the cow may not be reflected in an increase in the rate of beat of the heart.

It is of interest to note that the loss of body weight of the cows which were given 15 g. of iodinated casein was greater than the loss of weight of those which were given 30 g. This difference is related to the available food supply, for the pasture was of poor feeding value when 15 g. were given and in this experiment the control cows lost weight, while when 30 g. were fed the food supply at pasture was good. The difference in the loss of body weight in the two experiments suggests that, where the food supply is ample, a considerable part of the loss of body weight which results when an iodinated protein is given can be prevented.

#### THE INCORPORATION OF IODINATED CASEIN INTO CATTLE CUBES

For the purpose of increasing milk yields on commercial farms by feeding iodinated casein, the incorporation of iodinated casein into cattle cubes is essential. By incorporating iodinated casein in a cattle cube a very thorough mixing of the iodinated casein with suitable cattle foods can be made and this should ensure that the cube would be completely consumed in the majority of cases. At the same time the nutritive value of the cube could be adjusted so that if it was fed in addition to the normal ration of the cow, an adequate stimulation of milk production would be associated with an additional supply of nutrients to meet the increased requirement. Such an adjustment should prevent a serious loss of body weight, for it has already been deduced that when the food supply is ample the loss of body weight which occurs when iodinated casein is given, is smaller than when the supply of food is poor.

The manufacture of cattle cubes includes stirring of the finely ground ingredients in large metal containers, heating to not more than 130°F., the addition of molasses and forcing the mixture through metal dies. It was thought, therefore, that a partial destruction of iodinated casein might occur under such conditions, and, even following manufacture, the potency of the iodinated casein might deteriorate, especially if conditions of storage were bad. For these reasons additional experiments were carried out to determine whether a cube containing iodinated casein could be made which would be palatable to dairy cows; whether such a cube would prevent a loss of weight when fed as a supplement to normal rations; whether the manufacturing process would result in a loss of potency of the iodinated casein and whether the iodinated casein cube would retain its potency after storage under bad conditions.

It was decided that a daily dose of 20 g. of iodinated casein would be used, for the results of experiments at such a dose level would give additional information on the shape of the dosage-response curve. The additional nutrients to be supplied by

the cube were calculated, assuming that the dose of 20 g. would elevate the total metabolism of the cow by between 20 and 25%, and that the average level of production of the cows to be given the cube would be approximately 2 gallons of milk daily. The additional nutrient requirement thus estimated, a mixture of normal cattle foods was calculated and this supplied the additional requirement of energy and protein in a daily ration of 4 lb. The final composition of the cube follows.

Press extraction decorticated ground-nut cake	25 parts
Wheat feed	40 "
Molasses	10 "
Oats	20 "
Sugar-beet pulp	5 "
Minerals	2 "

The oil content of the cube was kept as low as possible, but no difficulty was found in forcing the ingredients through the cubing dies.

Two batches of the cube were manufactured. The first batch contained no iodinated casein, and part of this batch was ground to a fine meal. The second batch contained 11.2 kg. of iodinated casein per ton, so that the daily ration of 4 lb. would supply 20 g. of iodinated casein.

To test the palatability of the cubes, they were fed to some Ayrshire cattle which were known to be very fastidious in their food habits. Ten cows were used for the test, and although they all ate the control cube readily, refusals occurred when the iodinated casein cube was fed. At first the iodinated casein cube was eaten with relish, and no refusals occurred for the first 2 days. Subsequently one cow was off feed for two meals, one cow refused at one meal and a further cow refused all cubes following the second day. It can be concluded, therefore, that these cows could still detect the iodinated casein even when it had been thoroughly mixed with the cube ingredients, which by themselves were very highly palatable. The test with these highly fastidious cattle, however, indicates that the cube was not totally unpalatable, and that on the majority of farms an adequate intake of iodinated casein could be assured.

To determine whether deterioration of the potency of the iodinated casein during manufacture or following storage in cube form had occurred, a larger scale experiment was carried out with thirty-six dairy cows. Three farms co-operated in the experiment, and on each farm the cows were divided into blocks, each containing three comparable animals. There were three experimental treatments to which the cows in each block were allocated by random methods.

1. 'Control treatment', consisting of normal feeding and management throughout the experiment.

2. 'Meal plus iodinated casein treatment', consisting of the same normal feeding and management, but with the daily addition of 4 lb. of control cube and 20 g. of iodinated casein for a period of 21 days. The control cube was finely ground and the iodinated casein was mixed with this meal immediately before feeding.

3. 'Iodinated casein cube treatment', consisting of the same normal feeding and management as for the control treatment, with the addition of 4 lb. of the iodinated casein cube.

The experiment was divided into two parts. The first part was carried out so that treatment commenced at the beginning of the third week of November 1943, immediately following the manufacture of the iodinated casein cube. The second part was carried out following storage of the cube for 5 weeks under rather damp conditions on the farm, and in this part of the experiment, treatment commenced during the last week of December 1943. Each of these two parts of the experiment was divided into three periods, an initial period of 2 weeks, an experimental period of 3 weeks and a final period of 3 weeks.

The normal rations on which the experimental treatments were imposed varied from farm to farm, but in each case all cows were fed according to their milk production. During the first part of the experiment there was a change in the feeding of all cows from autumn to winter rations, but during the second part of the experiment the rations remained unchanged throughout.

### Results

Some slight difficulties were met in persuading the cows to consume the additional iodinated casein, whether it was in cube form or as an addition to the control meal. Generally, however, the meal was less palatable than the cube, and it was only

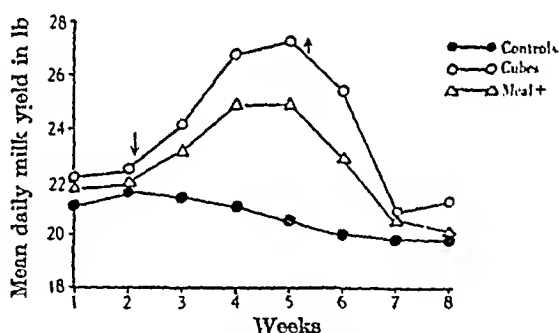


FIG. 5. The effect of the incorporation of iodinated casein into a cattle cube on its activity as judged by responses in milk yield.

Table 18. *Mean milk yields in lb. per day*

Part	Treatment	Initial period		Experimental period			Final period		
		Week 1	Week 2	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
1	Controls	19.3	19.8	20.0	19.5	18.9	19.0	18.8	19.7
	Meal	21.0	21.3	23.6	24.0	24.1	20.8	19.4	20.1
	Cube	20.1	20.2	22.3	24.1	24.7	21.9	18.8	20.1
2	Controls	22.3	22.8	22.4	22.1	21.7	20.8	20.6	20.1
	Meal	22.8	22.9	24.0	25.8	25.7	24.7	21.7	20.5
	Cube	23.7	24.2	25.4	28.6	29.1	28.3	22.4	22.1
Both parts	Controls	21.1	21.6	21.4	21.0	20.5	20.1	19.8	19.9
	Meal	22.1	22.2	23.4	25.1	25.1	23.1	20.8	20.3
	Cube	22.2	22.5	24.1	26.8	27.2	25.5	20.9	21.3

possible to ensure an adequate consumption in the case of some cows by mixing the meal and iodinated casein with silage or with mangels. Food refusals were greatest on the farm where the feeding level of the cows was highest, and on this farm one

cow constantly refused her ration of meal. The results from this cow have therefore been discarded in the analysis of the results.

Milk yields were recorded twice daily throughout the experiment to the nearest quarter of a pound. Table 18 shows these milk yields summarized according to the time at which the experiment was conducted. Fig. 5 shows the mean milk yields during both parts of the experiment.

It can be seen that milk yields increased appreciably when iodinated casein was given, and that when feeding stopped milk yields returned to normal. For the purposes of statistical analysis the changes in milk yield of each cow from the initial period to the last 2 weeks of the experimental period were calculated and analysis of variance then applied to these changes. The yield of the cow which refused her ration was calculated by the missing plot technique [Yates, 1933] and the complete analysis is shown in Table 19.

Table 19. *Analysis of variance of changes in milk production*

Component	Degrees of freedom	Mean square	F
Total	35	—	—
Part 1 versus part 2	1	0.35	785.0*†
Farms in parts of experiment	2	911.11	3.36 N.S.
Blocks on farms	8	346.86	1.26 N.S.
(a) Iodinated casein versus no iodinated casein	1	10,146.00	36.80**
(b) Cube versus meal	1	679.47	2.46 N.S.
Interaction— <i>a</i> × parts of experiment	1	15.45	17.84 N.S.†
Interaction— <i>b</i> × parts of experiment	1	141.19	2.12 N.S.†
Residual = error	19‡	275.64	—

\* Significant when  $P=0.05$ .

\*\* Significant when  $P=0.01$ .

† Error variance the greater variance.

‡ One degree of freedom subtracted for missing yield.

N.S. Not significant.

The significance of each of the comparisons which the experiment was designed to show is indicated in the last column of the table. First, iodinated casein feeding stimulated milk production; secondly, there was no difference in the response in the two parts of the experiment; thirdly, there was no differential effect between feeding iodinated casein as the cube and in the form of the meal, and, lastly, there was no difference in the response to the meal compared with the cube in the two parts of the experiment. These results show that there is no loss of potency of iodinated casein following the manufacturing process or following storage for a short period under farm conditions.

As there was no statistically significant difference between the mean yields of the cows which received the iodinated casein in the two different forms, or in the two parts of the experiment, the responses of the individual cows can be used for a further study of the factors influencing the variation in the response of the individual to iodinated casein feeding. There was a far larger variation in the response of the cows to iodinated casein than in the cows shown in Table 8. Part of this may have been due to the fact that experimental errors were slightly higher, but all the variation cannot be explained in this way. One cow increased in yield from 21.5 lb. daily to a maximum of 36.8 lb., an increase of 71 %, while another only increased in yield by 0.9 %. Table 20 shows the mean increases in yield of the cows arranged in descending

order of percentage response together with data on their productive capacity as measured by their peak yields in early lactation, and their 'stage of lactation' as measured by the ratio of the yield of milk they were giving at the commencement of treatment to their peak yields.

It will be noted from Table 20, that the cows which showed the greatest percentage increase were those which had declined in yield from the peak to the greatest extent, while those cows which were producing over 90 % of their peak yield when the experiment commenced did not respond to such a considerable extent. The summary at the foot of Table 20 has been made by dividing the data into four groups in descending order of the percentage response in milk production.

Table 20. *Mean daily increases in yield and mean percentage increase in yield during the 7th–21st days of treatment, when 20 g. of iodinated casein were fed*

Cow no.	Peak yield	Lactation stage	Response		Cow no.	Peak yield	Lactation stage	Response	
			lb.	%				lb.	%
1	61.0	35	14.3	66.4	13	40.0	61	4.4	17.9
2	36.6	30	5.5	50.3	14	47.0	50	4.1	17.6
3	57.0	30	5.4	31.7	15	38.7	51	4.3	16.9
4	33.0	74	3.7	31.1	16	26.6	98	4.3	16.9
5	33.4	67	6.7	29.6	17	30.0	95	4.2	14.5
6	43.0	48	5.4	27.2	18	28.0	72	2.8	13.7
7	19.4	62	5.2	26.5	19	54.6	47	2.8	11.2
8	46.3	38	4.2	24.0	20	26.6	98	2.4	10.5
9	49.4	56	6.6	23.8	21	21.7	92	1.5	7.6
10	34.0	81	6.2	22.5	22	26.7	99	0.8	3.0
11	29.7	75	4.4	19.9	23	28.1	97	0.2	0.9
12	36.3	60	4.2	19.6					

#### Summary

Group 1.	% response over 26.5	44.0	47.0	6.8	39.4
Group 2.	% response 17.9–26.5	35.9	62.0	5.1	22.7
Group 3.	% response 11.2–17.9	35.0	71.2	4.7	16.2
Group 4.	% response 0.9–11.2	30.5	88.1	1.4	5.5

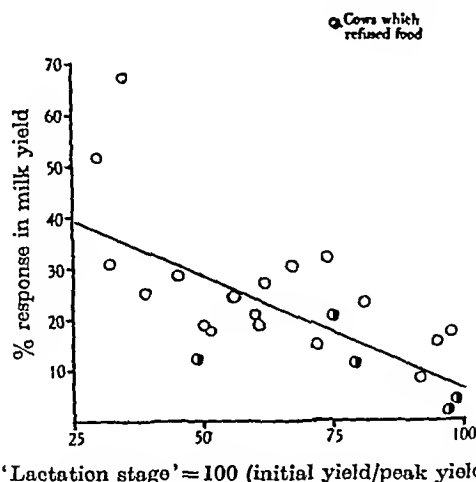


FIG. 6. The relation between 'Lactation Stage' and the increase in milk yield when iodinated casein is fed.

The large variation in the percentage response in Table 20 is obviously related to the 'stage of lactation' of the cow, but, at the same time, it should be noted that

there was an association of the stage of lactation of the cow with the cow's productivity at the peak of lactation, that is, in this sample those cows which were relatively late in lactation, as judged by the ratio peak/initial yield, tended to be those which were the heaviest producers. To ascertain the importance of the peak yield or productive capacity of the cow in determining the response to a standard dose of iodinated casein, a study of the data in Table 20 by multiple and partial correlation methods was made. This showed that the 'stage of lactation' was the most important factor determining the variation in the response, and that when the 'stage of lactation' was held constant at a mean value, there was but little variation in the response which could be associated with a variation in the initial productive capacity of the cow. Thus, from this limited amount of data it is possible to conclude that the greater the divergence of the yield of the cow immediately prior to treatment, from the yield she is capable of giving during the early part of her lactation, then the greater will be the response which she gives to iodinated casein feeding providing she has not reached the phase of rapid terminal decline in yield. The difference between the response of cows initially yielding the same quantity of milk, may therefore be large, and can be explained in terms of differences in their capacity for milk production as expressed by their peak yields in early lactation. The cow which has given the greatest yield at that time will then be at a relatively later 'stage of lactation' immediately before treatment begins.

The fat percentages of the milk were determined for 21 of the cows, both in the initial period and in the last week of the experimental period. There was a net increase in the fat percentage of the cows which received the iodinated casein of 0.19, but this was not significant statistically. Body weights were recorded on two of the co-operating farms and estimates were also made of the condition of each cow before and after treatment on all farms. These data are shown in Table 21.

Table 21. *Mean body weights and mean 'condition scores'*

Treatment	Body weight in lb.*			Score on a scale 0-10†		
	Initial	Final	Change	Initial	Final	Change
Controls	1079.6	1115.1	+35.5	6.5	6.8	+0.3
Meal	1056.4	1075.6	+19.2	6.5	6.4	-0.1
Cube	1050.8	1080.2	+29.4	6.7	6.1	-0.6
	* 21 cows.			† 30 cows.		

Statistical analysis of the changes in body weight showed that the slight net fall in body weight was not statistically significant. Only on one farm was a loss of body weight apparent in treated animals and on this farm the plane of nutrition was lower than on the other. The net loss of weight on this farm was 42.6 lb. during the period of 3 weeks. The slight changes in the condition of the cows were not statistically significant, and, on the scale of points which was used, a fall of 0.6 unit represents a very small change in body condition. The co-operating farmers, however, agreed that a slight loss of condition had occurred in some cows, and in others the loss of condition had been quite marked.

Each cow was examined before treatment and again after treatment, while the herdsmen were instructed to watch carefully for any abnormalities which occurred. Slight symptoms of an increased metabolism were noticeable at each farm. The

treated cows were more alert and nervous, their heart rates were higher than those of the comparable control cows, there was slight sweating in the treated cows and in one case slight frothing at the mouth. Cow 1, which gave a maximum response of 71 % during the last week of treatment, was normal throughout the experimental period, save for some tachycardia and slight sweating. This cow lost some weight and condition and her respiration was heavy on a number of occasions.

The results of the experiment show that the incorporation of iodinated casein into a cattle cube is of considerable value in ensuring an adequate consumption of iodinated casein, for, compared with mixing with a meal, cube feeding results in a smaller incidence of food refusals, this reflecting the general preference of cows for cubes as opposed to meals. The cubing process, and the subsequent storage of the cubes under the conditions normally prevailing on farms results in no loss of potency of the iodinated casein, while it appears that by feeding the cube as a supplement to normal rations, a serious loss of body weight and body condition can be prevented, although cows given the additional allowance tended to lose some weight and condition.

The mean percentage response in milk production in the second week of treatment to this dose of iodinated casein was 21.9. The results of the three dose levels of iodinated casein are summarized in Table 22.

Table 22. *The responses in milk production in the second week of treatment and the effect on the cow when three doses of iodinated casein were given*

Dose of iodinated casein	Batch no.	% increase in milk yield	Palatability	Symptoms of	
				Hypermetabolism	Iodism
15	NC3	16.7	Excellent	Very slight	Nil
20	NCB1+2	21.9	Fair	Slight	Nil
30	NC3	32.7	Fair	Very pronounced	Slight

Tadpole assays on iodinated casein NCB1+2 showed that it did not differ in potency from iodinated casein NC3 [Deanesly & Parkes, 1945], and it was a member of the same series of preparations [Pitt Rivers & Randall, 1945]. The table thus shows that the response to iodinated casein is directly proportional to dosage, and a consideration of the data on palatability, and the incidence of symptoms of hypermetabolism and iodism, suggest that the optimal dose of an iodinated protein is one that elevates milk production by 20 %.

#### DISCUSSION OF RESULTS WITH REFERENCE TO THEIR PRACTICAL APPLICATION

The main object of this experimental work with iodinated proteins has been to find whether the stimulation of the milk yields of dairy cows by feeding iodinated proteins can be used to increase the milk production of commercial farms having special regard to any possible effect of such a stimulation on the health and well-being of the cows involved. On the basis of the data which have been reported in this and the preceding paper [Blaxter, 1945] it is possible to draw a number of conclusions relating to the application of iodinated protein treatment to dairy cows.

The experiments with iodinated casein have established that an increase of milk yields by 20 % is near the optimal level of stimulation, for at such a dose level severe

symptoms of hypermetabolism do not arise and the behaviour and normal physiological processes of the cow are not unduly disturbed. The loss of body weight is still apparent when small doses of iodinated casein are given, but it has been shown that an additional supply of food amounting to 20% of the normal nutrient requirements prevents the occurrence of a considerable part of the loss in body weight.

To express the dosage of an iodinated protein which will result in a 20% stimulation of milk yield, it is necessary to express the potency of the materials which have been used in terms of a standard preparation. Iodinated casein NC3 is probably the most reliable preparation to use for this purpose, but in referring the activity of other preparations to this preparation, it must be remembered that there must be considerable errors attached to these estimates, for the percentage responses to each of the iodinated proteins have been determined in separate experiments. Table 23, however, shows the relative potencies of the preparations expressed in this way.

Table 23. *Potency of iodinated protein preparation per g. in terms of the standard iodinated casein NC3, and the chemical composition of these materials*

Preparation	Dosage g.	Response in milk yield %	Potency per g. when iodinated casein NC3 = 100	Iodine content*	
				Total	Acid- insoluble
Iodinated casein NC1	30	0.0	0.0	6.3	1.2
Iodinated casein NC2	30	29.8	90.5	7.3	1.2
Iodinated casein NC3	30	32.8	100.0	9.0	2.8
Iodinated casein NCB1 + 2	20	21.9	99.9	7.7	1.6
Iodinated Ardein N4MB	50	27.1	49.5	5.8	0.88
Iodinated Ardein N9 + 10MB	50	5.0	0.9	5.5	0.31
Iodinated Ardein LXM	120	19.7	15.0	6.2	0.66
Iodinated ox plasma N4	200	15.1	6.8	5.4	0.4

\* I am indebted to Mrs Pitt Rivers and Mr Randall for this information.

A comparison of the large range of the potencies of the preparations with their 'thyroxine' iodine content shows little agreement. Some of this may be due to the large sampling errors which were met in determining the iodine content of the preparations, but it appears that chemical analysis alone will not be sufficient to express with any accuracy the dose of an iodinated protein which will stimulate milk production by 20%. Thus an initial biological assay appears essential to determine potency.

In using iodinated protein on commercial farms, a consideration of the type of animal to be treated must be made. As it has been found that stimulation of the metabolism in early lactation by feeding iodinated casein has little effect on milk production, treatment at this time is not desirable, while in the later part of lactation the stimulation of the milk yield of cows which are pregnant has also been shown to be an undesirable procedure. In any case lactation cannot be prolonged when the phase of terminal decline has been reached.

The cows which should be fed iodinated protein are therefore those which have passed the peak of their lactation and which are not heavy in calf. Of these cows the greatest response will generally be given by those which have declined in yield from their peak to the greatest extent, providing that they have not reached the phase of rapid termination of lactation.



The optimal period of stimulation is difficult to establish from the data available, but the results of the experiments in which a large dose of iodinated Ardein was given for a long period suggest that 1-2 months' treatment is probably a practical possibility.

#### SUMMARY

1. Experiments have been carried out using preparations of iodinated casein, designed to elucidate the factors responsible for the variation in the response to iodinated protein feeding, and to find whether iodinated proteins can be used to increase milk production under practical conditions.

2. Of three preparations of iodinated casein fed to dairy cows, two were active and one was not. This result was in substantial agreement with the results of assays with tadpoles.

3. Coating iodinated protein with solid stearic acid and variation in the basal ration of the experimental cows were without any effect on the potency of the preparation. Both heifers and cows, and Guernsey and Shorthorn cattle, responded to treatment with iodinated casein.

4. The response to an iodinated protein is intimately related to the stage of lactation of the cow. In early lactation the response is small, and at the end of lactation it is not possible to elicit a response. In mid-lactation the percentage response increases as lactation declines, while the greater the initial yield the greater the response in pounds per day. The high yielding cow—as measured by her productivity in early lactation—responds very markedly when her yield has fallen to 2 gal. a day while the low yielding cow does not. This is in reality a 'lactation stage' effect, and the effect of the stage of lactation on response has been confirmed by experiments with the same cow. Preliminary data suggest that the same relationship applies to fat secretion, and it can be interpreted in terms of the amount of mammary tissue present and its state of functional activity.

5. The mean percentage response in milk production has been shown to be directly proportional to dosage within a range of stimulation from 16 to 33 %. The symptoms of hypermetabolism are much more severe at the higher dose levels than would be expected on the basis of a directly proportional relationship. It has been suggested, therefore, that the optimal stimulation should be in the region of 20 %.

6. Iodinated casein has been incorporated in a cattle cube which was consumed satisfactorily, and it has been shown that the manufacturing process and the storage of iodinated protein cubes does not result in a reduction of the galactopoietic potency of the iodinated casein they contain.

7. A considerable part of the loss of weight which occurs when iodinated casein is given has been shown to be prevented when the cow is given an additional supply of nutrients. A light loss of body condition was still observed.

8. The relative galactopoietic potencies of the various samples of iodinated protein which have been used have been expressed using iodinated casein NC3 as a standard, and the practical possibility of the iodinated protein stimulation of milk yields is briefly discussed.

My thanks are due to the members of the Iodinated Protein Group of the Agricultural Research Council for their continued advice and guidance in carrying out

this work. I am also indebted to Prof. R. Rae, Mr J. Hunter-Smith and Mr J. C. Robinson for their kind co-operation in the experiment with iodinated casein cubes, and to Mr R. W. Eccles and Mr F. C. Hammond, students of the University of Reading and of the Royal Veterinary College, for helping to collect some of the data during their vacations.

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## APPENDIX

Table 1. *Details of experimental cows*

No.	Block	Treat- ment	Breed	No. of times calved	Date of calving	Date of service
1	1	B	S	2	31 Oct. 1942	23 Jan. 1943
2	2	B	S	1	5 Feb. 1943	9 May 1943
3	3	B	S	4	22 Sept. 1942	19 Dec. 1942
4	4	B	G	2	11 Feb. 1943	2 Apr. 1943
5	1	C	S	2	15 Jan. 1943	14 Mar. 1943
6	2	C	S	1	10 Mar. 1943	27 May 1943
7	3	C	S	3	23 Sept. 1942	28 Apr. 1943
8	4	C	G	2	30 Jan. 1943	26 Apr. 1943
9	1	A	S	2	9 Jan. 1943	1 July 1943
10	2	A	S × G	1	7 Dec. 1942	27 Jan. 1943
11	3	A	S	5	21 Sept. 1942	11 Feb. 1943
12	4	A	G	1	1 Feb. 1943	6 May 1943
13	1	E	S	2	8 Dec. 1942	16 Feb. 1943
14	2	E	S	1	21 Jan. 1943	10 Apr. 1943
15	3	E	S	2	14 Sept. 1942	24 Feb. 1943
16	4	E	G	5	20 Feb. 1943	11 June 1943
17	1	D	S	2	6 Dec. 1942	13 Feb. 1943
18	2	D	S	1	30 Nov. 1942	6 Mar. 1943
19	3	D	S	2	7 Nov. 1942	28 Jan. 1943
20	4	D	G	4	25 Mar. 1943	17 May 1943

Table 2. *Mean weekly yields of milk in lb. per day*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	23.3	23.7	24.0	23.5	21.7	19.2	18.0	16.8	15.3	13.6
B	22.7	21.9	22.5	21.8	21.9	23.2	22.0	19.3	14.6	12.5
C	22.9	23.2	23.4	23.0	23.7	26.2	26.1	21.9	15.7	15.9
D	22.6	22.0	23.5	20.3	19.6	18.4	17.5	16.8	15.7	15.5
E	22.9	22.1	23.5	21.4	22.5	24.3	23.7	20.3	15.3	15.5

\* Treatment. A, Control at pasture; B, Coated NC3 at pasture; C, Uncoated NC3 at pasture; D, Control winter feeding; E, Coated NC3 + winter feeding.

† Weeks. 1-3, Outdoor control; 4, Indoor control (D and E); 5-7, Experimental; 8-10, Final control.

Table 3. *Mean fat percentages of the milk*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	3.88	4.12	4.36	4.36	4.52	4.51	4.82	4.65	4.83	5.28
B	3.82	4.00	4.22	4.21	4.09	4.53	4.76	4.98	4.62	4.69
C	3.34	3.54	3.87	3.86	3.63	4.27	4.32	4.55	4.18	3.87
D	3.79	3.83	4.13	4.23	4.43	4.46	4.52	5.04	4.84	4.75
E	3.64	3.65	3.89	4.02	4.00	4.86	4.76	5.14	4.44	4.14

\* † As in Table 2.

Table 4. *Mean fat yields per week in pounds*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	6.13	6.74	7.18	6.98	6.72	5.91	5.89	5.23	5.12	4.52
B	6.05	6.09	6.60	6.38	6.17	7.29	7.28	6.29	4.50	3.61
C	5.43	5.83	6.51	6.29	6.11	7.99	7.98	7.00	4.62	4.34
D	6.19	6.10	6.97	6.14	6.24	5.84	5.63	6.00	5.42	4.90
E	6.02	5.79	6.64	6.29	6.49	8.47	8.04	7.45	4.76	4.56

\* † As in Table 2.

Table 5. *Mean solids-not-fat percentage in the milk*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	9.19	9.18	9.15	9.06	8.97	8.94	9.08	9.16	9.03	8.79
B	9.27	9.15	9.15	9.08	9.01	9.06	8.95	9.09	8.91	9.19
C	8.93	8.89	8.87	8.72	8.65	8.69	8.75	8.80	8.65	8.64
D	9.06	9.05	9.05	8.80	9.08	9.11	9.21	9.23	9.29	9.32
E	8.93	8.85	8.93	8.74	8.74	8.82	8.88	8.93	8.90	8.82

\* † As in Table 2.

Table 6. *Milk phosphatase (King and Armstrong units) per ml.*

Days	Preliminary control					Experimental				Final control			
	11	14	18	25	28	4	7	14	20	4	7	11	18
A*	125	109	128	125	118	134	156	188	223	242	207	198	192
B	186	156	179	172	155	101	74	69	69	106	230	220	238
C	193	181	189	197	187	112	70	70	65	104	254	273	197
D	143	147	160	161	173	215	213	191	221	216	175	157	206
E	185	166	189	190	187	112	67	73	65	113	210	230	183

\* Treatments as in Table 2.

Table 7. *Mean heart rates per minute*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	64.4	63.7	67.1	69.2	65.8	61.6	60.1	60.5	57.5	54.9
B	68.7	67.5	72.9	74.0	78.4	81.7	81.8	71.4	59.6	55.5
C	65.8	65.6	69.2	70.5	73.0	80.5	81.6	70.3	56.4	52.5
D	67.4	66.0	70.5	63.1	63.9	63.9	64.2	65.3	65.5	65.3
E	68.0	65.5	70.6	63.1	71.5	80.7	84.0	75.1	62.7	61.6

\* † As in Table 2.

Table 8. *Mean number of respirations per minute*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	28.3	29.6	30.9	28.3	29.3	28.2	28.8	27.6	29.3	26.3
B	31.0	32.5	34.4	33.5	37.8	38.3	41.1	36.3	32.2	29.8
C	28.6	28.7	30.9	28.9	31.0	31.5	36.6	28.7	28.4	26.0
D	29.1	30.1	32.3	29.7	30.3	28.6	31.4	31.3	32.5	30.4
E	30.5	30.3	32.3	28.6	30.4	32.3	38.5	34.0	33.0	31.7

\* † As in Table 2.

Table 9. *Mean rectal temperature in °F.: add 100.0° F. to each reading*

Treat- ment*	Week†									
	1	2	3	4	5	6	7	8	9	10
A	0.71	0.81	1.01	0.93	0.84	0.90	0.85	0.96	1.12	1.08
B	0.85	0.81	1.02	1.05	1.13	1.18	1.12	1.15	0.90	0.97
C	0.92	0.88	1.09	1.02	1.02	1.09	1.19	0.97	0.87	1.04
D	0.62	0.67	0.77	0.93	0.84	0.97	1.11	1.02	0.99	1.05
E	1.03	0.82	0.93	1.02	1.07	1.14	1.39	1.17	0.97	1.04

\* † As in Table 2.

# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 5. THE EFFECT ON BASAL METABOLISM OF MILK FROM COWS FED WITH IODINATED PROTEIN

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(Received 13 October 1944)

### THYROXINE IN MILK—REVIEW OF LITERATURE

The credit for first clearly establishing that iodine is a normal constituent of milk goes to Maurer & St Diez [1926]. Their findings have since been confirmed by Kieferle & Kettner [1927], McClendon, Remington & von Holnitz [1930], Scharrer & Schwaibold [1927] and Harvey [1935] and others. In both lactating women and cows Maurer & St Diez [1926] found that the iodine content of the colostrum was high and in the region of  $36\mu\text{g. per } 100\text{ ml.}$  By the second day of the puerperium the concentration had fallen to  $3\mu\text{g. per } 100\text{ ml.}$ , and thereafter remained at this figure  $\pm 1\mu\text{g. per } 100\text{ ml.}$  throughout lactation. The iodine content of milk varies according to the iodine intake. Scharrer & Schwaibold [1928] found that the milk of cows and sheep fed in meadows subject to flooding by sea water had an iodine content 300–800 % higher than that of animals fed in Upper Bavaria and Switzerland. The findings of McClendon *et al.* [1930] were similar. Magee & Glennie [1928] found that pasteurizing and boiling milk caused an iodine loss of 20 and 26 % respectively. Fellenberg [1926] found a greater proportion of iodine in the organic form in milk. Elmer [1938] extended this work, and using a modification of Leland & Foster's [1932] method found that thyroxine constituted barely 13 % of the total iodine secreted in milk. Schepps & Elmer [1935] showed, using the method of Harington & Randall [1929], that 50 % of the total iodine in the thyroid gland of new-born infants was thyroxine. From these findings Elmer concluded that the new-born child synthesized all the thyroxine it required.

The purely clinical observations on the therapeutic properties of maternal milk in providing an extraneous source of the thyroid hormone are conflicting. Herman [1914], Gordon [1922], and Dorff [1934] agree that the mother's milk does not contain substances which can counterbalance an infantile thyroid deficiency. Each author described the development of infantile myxoedema or cretinism in breast-fed babies. Dorff's observations in particular were striking. He reported on two families of breast-fed twins. In each case one twin developed infantile myxoedema and the other did not. On the other hand, Schein [1895] has stated that symptoms of myxoedema do not appear in athyrotic infants as long as they are breast-fed. Schein's view has received support from other workers who noted the effect of thyroid administration to the mother on the breast-fed child. Mossé & Cathala [1898a, b] treated with thyroid a mother with goitre who was nursing a goitrous child. The treatment cured not only the mother but also the child. Bramwell [1899] saw

the development of thyrotoxicosis in a suckling whose mother was being treated with thyroid. The symptoms disappeared when the mother stopped taking the thyroid. Neurath [1928] found that sucklings of mothers suffering from chronic constipation also suffered from constipation, and that the administration of thyroid to the mother cured the complaint in both mother and child. This problem was studied experimentally by Lukacs [1930]. By thyroid feeding he produced hyperthyroidism in lactating rats and he then compared the weight curve of the litter-rats with that of control animals. The young of the 'hyperthyroidized' rats were 31-54% lighter than the control animals. Lukacs's work has been attacked by Konsuloff [1932], who maintained that Lukacs failed to take into account the effect of thyroid on milk production. As thyroid feeding increases milk production and milk fat, Lukacs's observations are all the more valuable as he was able to demonstrate an impaired weight gain in young rats who were probably having a higher calorific intake than the control litter. Konsuloff, however, did not deny that the thyroid hormone is secreted in the milk and in a later publication [1935-6] he brought forward additional evidence in support of Lukacs's views. He found that the administration of thyroid to the mother produced an increase in the production of carbon dioxide in the suckling animal. These clinical and experimental observations all indicate that the thyroid hormone is secreted in the milk, and that changes in thyroid activity of the mother are reflected in the thyroxine content of her milk.

In 1934 Graham [1934a] reported that thyroidectomy in cows caused a diminution in milk secretion and milk-fat production, and that the addition of thyroid to the diet of normal or thyroidectomized cows caused a rapid rise in the yield of milk and milk-fat. In a later publication [1934b] he showed that this action was due to thyroxine. Folley & White [1936] confirmed Graham's work. They showed that if cows received injections of 10 mg. of thyroxine daily the milk production rose 28%. It is interesting to note that as early as 1899 Hertoghe drew attention to the fact that thyroid feeding in lactating women increased the size of the mammary gland and the milk secretion. He gave a cow small amounts of thyroid for 20 days, during which time the daily yield of milk rose by 15%. Hertoghe's monograph was later translated into German by Spiegelberg [1900]. Hertoghe again drew attention to the lactogenic properties of the thyroid hormone some years later [1915]. In a further reference to Hertoghe's work by Lukacs [1930], which at present I cannot trace, it is stated that with large doses of thyroid the daily milk yield of a cow was increased by 36%. Siegmund [1910] applied Hertoghe's observations with some success to lactating women deficient in milk production. Recently, Ludwig & Mutzenbecher [1939] isolated thyroxine from preparations of iodinated proteins. Such iodinated proteins, when fed to lactating mammals, produced an increase in the milk and the milk-fat production.

#### PRESENT STUDY

The present inquiry is to determine whether milk, from cows fed iodinated proteins, contains the thyroid hormone in excessive amounts, using the basal metabolism of milk-fed subjects as a guide.

## EXPERIMENTAL METHODS

A study was made of twenty-seven normal healthy nurses who had volunteered for the investigation. Their average age was  $21\frac{1}{2}$  ranging from 19 to 33. Observations were made on the basal metabolism, basal pulse rate, blood pressure under basal conditions, and body weight. The latter measurement was taken on all occasions in the morning after the determination of the basal metabolism, without shoes and wearing the same nursing clothes without the usual pocket contents. The determination of the basal metabolism was made by the closed-circuit method, the accuracy of which has already been reported [Robertson, 1937]. The apparatus used was the Benedict-Roth with recording kymograph. All machines have been alcohol-checked [Barrett & Robertson, 1937], but in addition each machine is checked once per week by a model whose basal metabolism is in the region of 56 cal. per hour.

## RESULTS

In Table 1 are given the effects of the ingestion of 1 quart of milk daily from cows fed iodinated protein on the basal metabolism, basal pulse rate, body weight, and blood pressure under basal conditions. It will be seen that no significant rise in the basal

Table 1. *Effect of the daily ingestion of 1 quart of experimental milk on twenty-seven normal female nurses*

	Mean values $\pm$ S.E.		
	Before	At end of 2nd week	At end of 4th week
Heat output (cal./sq.m./hr.)	$33.84 \pm 0.39$	$33.89 \pm 0.34$	$33.47 \pm 0.27$
Basal pulse rate per min.	$68 \pm 1.5$	$67 \pm 1.4$	$66 \pm 1.4$
Weight in lb.	$125\frac{1}{2}$	$127\frac{1}{2}$	$127\frac{1}{2}$
Blood pressure under basal conditions (mm. Hg)	100/55	105/60	100/60

metabolism occurred at the end of 2 weeks ( $t=0.23$ ,  $P=0.8$  [Fisher, 1938]). At the end of 4 weeks the basal metabolism fell slightly but not significantly ( $t=1.9$ ,  $P=0.1$ ). The basal pulse rate and blood pressure also remained unchanged throughout the observation period. There had, however, been a significant gain in weight by the end of the second ( $t=4.6$ ,  $P<0.001$ ) and fourth weeks ( $t=4.7$ ,  $P<0.001$ ).

## DISCUSSION

From a study of the literature there would appear to be little doubt that the thyroid hormone is secreted in milk. It is probable, however, that the amount secreted is insufficient to act as the sole source of available thyroxine for the suckling. Clinical and experimental investigations appear to agree that if the mother or nursing mammal is fed on thyroid some is excreted in her milk, which may produce toxic symptoms of thyroid overdosage in her sucklings.

It has been known for some time that the administration of thyroid increases the production of milk and milk fat. More recently it has been shown that a similar effect can be produced by the ingestion of iodinated protein, apparently owing to its thyroxine content. While the administration of thyroid or iodinated protein

produces an increase in the yield of milk and milk fat, it also must result in the secretion of a milk with a higher thyroxine content than normal. The finding in this present investigation is that the ingestion of 1 quart of milk daily, from cows fed on iodinated protein, has no effect on the basal metabolism of normal healthy adults. It would appear therefore that although this specialized milk very probably contains an increased thyroxine content this is not sufficient to raise the basal metabolism of healthy adults.

A review of the literature suggests that caution is advisable in giving such a milk, as described above, to young infants, because there is evidence that milk with a higher thyroxine content than normal can cause symptoms of thyrotoxicosis in sucklings. As pasteurization results in the loss of approximately 20% of the total iodine in milk, it would be interesting to determine whether this loss of iodine affects that part which is present as thyroxine.

#### SUMMARY AND CONCLUSIONS

1. The literature concerning the thyroxine content of milk is reviewed.
2. Over a period of 1 month the ingestion of 1 quart daily of milk from cows fed iodinated protein produced no elevation of the basal metabolism, pulse rate or blood pressure in twenty-seven normal healthy adults.
3. The ingestion of 1 quart of this milk daily produced a significant increase in weight, showing that the calorific intake greatly outweighs the effect caused by any metabolic stimulant which may be present in the milk.
4. The literature suggests that some precautions should be taken regarding the feeding of young infants with milk obtained from cows fed iodinated protein. Such a milk may contain thyroxine in excess of normal and produce toxic symptoms.

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\* In the absence of the authors, this paper was read by Cadot de Gassicourt—whose name, in error, often appears in the literature as being the author of the communication.

# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 6. FURTHER EXPERIMENTS ON THE RESTORATION AND MAINTENANCE OF GROWTH AFTER THYROIDECTOMY

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(Received 17 December 1943)

In an earlier publication Rowlands [1942] showed that the retardation of growth in young thyroidectomized rats can be overcome by the daily injection of a suspension of desiccated thyroid powder, of a solution of thyroxine, or by the implantation of a tablet of crystalline thyroxine. The daily injection of 2.5  $\mu$ g. of thyroxine maintained growth at a normal level for a period of 100 days; the amount absorbed over the same period from a 40 mg. tablet, which also fully maintained growth, was so small as to be undetectable by the methods used. This work has since been extended in two main directions: (1) by comparison of the rates of absorption of tablets of the mono- and the di-sodium salts of thyroxine with that of tablets of thyroxine itself, and (2) by investigation of the action of an iodinated protein (iodinated Ardein) on maintenance and restoration of growth, and of the possibilities of using the response for the biological assay of this and allied substances. In the latter respect the experiments were somewhat disappointing; although the ability of the iodinated protein to overcome the deficiency of growth caused by extirpation of the thyroid gland cannot be doubted, the use of the method as a means of assay presents difficulties which, at present, seem insuperable.

### MATERIAL AND METHODS

*Test animals.* Immature male rats weighing 40–50 g., from two stocks, an albino colony (Ronan stock) and a black hooded colony (Mill Hill stock), were thyroidectomized under Avertin anaesthesia as previously described. Growth was measured by twice-weekly weighings taken at a constant time in relation to feeding. Maintenance or restoration is expressed throughout by the formula  $\frac{T-t}{I-t} \times 100$ , where  $T$  = increase in weight of injected thyroidectomized rat during the period treated,  $t$  = gain in weight of uninjected thyroidectomized rat over same or similar period, and  $I$  = increase in weight of intact control rat over the same period.

It should be noted, however, that in experiments on the restoration of growth it is not clear whether growth should be compared with that of intact rats of the same age or of the same weight. The above calculation is based on growth in rats of the same age. If the calculation is based on growth in rats of the same weight, the restoration effect is reduced by about 20% on account of the greater rate of growth over the period in consideration.

*Substances used.* The effect of the following substances was examined.

(1) Thyroxine (B.D.H.), mono-sodium thyroxine (B.D.H.) and di-sodium thyroxine. All three substances were administered as tablets, with or without the

addition of cholesterol as excipient. They were implanted for 100 days in the left axilla at the time of thyroidectomy.

(2) Thyroid powder, B.P. (Boots Pure Drug Co. Ltd., Batch No. 13084) received from Dr S. J. Folley. This preparation contained 0.11% thyroxine iodine and a total iodine content of 0.237%. For injection the powder was suspended in water at pH 7.2-7.5.

(3) Iodinated Ardein. Two preparations were used: N4 SF and N4 MB, containing respectively 3.61 and 5.76% of total iodine and 0.5 and 0.88% of acid-insoluble iodine [Pitt Rivers & Randall, 1945]. Non-iodinated Ardein (I.C.I., Stevenston, Batch No. DT/A/630) was also used. For injection these substances were dissolved in water at pH 7.2-7.5.

*Administration.* In experiments on the maintenance of growth the substances were injected daily for a period of 3 months from the day of thyroidectomy. In restoration experiments an interval of 6 weeks elapsed, to ensure almost complete cessation of growth, before injections were started. Injections were continued daily for 7 weeks.

*Tablet making.* Disk-shaped tablets were made on a hand-press using a die 3 mm. in diameter. They were prepared by Mr F. H. Crisp.

#### MAINTENANCE OF GROWTH BY THYROXINE AND ITS SODIUM SALTS

*Thyroxine tablets without excipient.* The thyroid gland of the rats of the albino strain used in this work was usually found to be hyperplastic and its removal was often troublesome because of haemorrhage. The growth rate of these rats was slightly greater than that of the hooded rats used, whose thyroid glands were less hyperaemic. The growth-maintaining capacities over a period of 100 days, of one, two and three tablets of thyroxine, each weighing 10 mg., were compared in thyroidectomized rats from both colonies. The results are given in Table 1. The response elicited by the hooded rats implanted with one tablet was about equal to that produced in the albino rats implanted with two tablets. Complete maintenance of growth was secured in the hooded rat with three tablets, and in the albino rat with four tablets. No deleterious effects were produced in the albino rats by the implantation of twice as many tablets as were necessary for maximum growth. Assuming, therefore, that the rate of absorption of the tablets is the same in the rats from the two colonies, it is clear that those with hyperplastic thyroids require more thyroxine than those having a less active gland. In neither stock was a measurable amount of thyroxine absorbed in the period of 100 days.

All subsequent experiments were carried out on hooded rats as they were more plentiful and because of the greater ease with which thyroidectomy could be performed. A period of 100 days is insufficient to measure the rate of absorption of thyroxine implanted as a tablet. Consequently, another three hooded rats weighing 42 g. were thyroidectomized and implanted for a maximal period with three 10 mg. tablets of thyroxine. The average body weight of these rats was maximal at 250 g. about a year later and this weight was maintained with only a slight decrease until the end of the experiment. One of the three rats died after 480 days, and unfortunately was destroyed before the tablets were removed. Another died 603 days after implantation, and the third was killed in good condition on the same day.

Table 1. *Maintenance of growth in thyroidectomized rats by thyroxine and its salts administered as 10 mg. tablets implanted subcutaneously for 100 days*

Tablets implanted					Body weight g.		Main- tenance %
Substance	Wt. mg.	No. of tablets	No. of rats	Stock	Initial	Final	
Nil	10	0 (T.C.)*	5	Mill Hill	44	86	0
Thyroxine, no excipient		1	8		42	168	63
		2	11		43	182	72
		3	10		43	225	104
Nil		0 (I.C.)*	7		46	222	100
Nil	10	0 (T.C.)	4	Ronan	45	103	0
Thyroxine, no excipient		1	3		43	147	28
		2	4		47	208	62
		3	2		44	230	78
Nil		0 (I.C.)	4		44	267	100
Nil	10	0 (T.C.)	5	Ronan	45	91	
Thyroxine, no excipient		4	6		47	214	100
		6	2		46	190	81
		8	3		44	222	109
Nil		0 (I.C.)	5		46	214	100
Nil	20	0 (T.C.)	5	Mill Hill	44	86	0
Thyroxine 25%, cholesterol 75%		1	7		44	144	48
		2	7		44	146	50
		3	2		49	184	78
		4	2		47	186	81
Nil		0 (I.C.)	5		49	211	100
Nil	10	0 (T.C.)	5	Mill Hill	44	86	0
Mono-sodium thyroxine 25%, cholesterol 75%		1	7		46	181	66
		2	7		45	182	67
		3	7		44	197	78
Nil		0 (I.C.)	9		41	225	100
Nil	10	0 (T.C.)	4	Mill Hill	46	84	0
Di-sodium thyroxine 25%, cholesterol 75%		1	12		46	194	91
		2	10		43	196	93
		3	13		43	200	98
Nil		0 (I.C.)	8		47	206	100

\* T.C. = thyroidectomized controls; I.C. = intact controls.

The amount of thyroxine absorbed from each tablet was about 1 mg., and even after such a prolonged period of implantation they appeared indistinguishable from similar unimplanted tablets of the same size and shape.

*Thyroxine tablets with excipient.* To reduce the amount of thyroxine implanted it was found necessary to prepare tablets in which the active substance was diluted with cholesterol. The smallest tablet containing 25% thyroxine and 75% cholesterol that could be prepared weighed 20 mg. Unfortunately this allowed only a halving of the smallest amount of thyroxine previously given and which was shown above to effect maintenance of growth to the extent of 63% of the normal. One, two, three or four tablets of this composition were implanted for 100 days. No difference was found between the effects produced by the implantation of one and two tablets, but the results obtained in the main agree well with those given above by the unadulterated tablets implanted into the same stocks of rats. No detectable loss in tablet weight occurred during the implantation period.

*Tablets of mono- and di-sodium thyroxine.* Tablets weighing 10 mg. and containing 25 % of thyroxine salt and 75 % cholesterol were used. One, two or three tablets of each salt were implanted for 100 days into thyroidectomized rats; their effects on body growth are shown in Table 1. One tablet of di-sodium thyroxine is more effective than three tablets of the mono-sodium salt. No detectable loss in weight of the mono-sodium thyroxine tablets occurred during the 100 days they were implanted, but the average amount absorbed from each tablet containing the di-sodium salt was approximately 1 mg. It can be seen further, that the mono-sodium salt of thyroxine is, on the average, about three times as effective as thyroxine itself, and because the di-sodium salt is at least three times more active than the mono-sodium compound, the growth-maintaining activities of the three substances, thyroxine acid, its mono-sodium salt and its di-sodium salt, are probably in the ratio of 1:3:10.

#### MAINTENANCE AND RESTORATION OF GROWTH WITH IODINATED PROTEIN

The capacity of an artificial 'thyroprotein', prepared by iodination of skimmed milk, to cause growth in young thyroidectomized goats was described by Reineke & Turner [1941], who suggested that the reaction might be used as a means of assay of substances having thyroid-like activity. Since, however, complete restoration of growth is seen in thyroidectomized rats treated with thyroid preparations and thyroxine, an examination in this species of the growth-promoting capacity of iodinated proteins seemed desirable. Two preparations of iodinated Ardein (N4 SF and N4 MB) were used.

*Maintenance of growth with iodinated Ardein N4 SF.* Twenty young thyroidectomized rats were injected daily for 12 weeks with 1 mg. of iodinated Ardein N4 SF. An equal number of intact rats of the same weight was injected similarly. Their body weights at the end of the injection period are shown in Table 2.

Table 2. *Maintenance of growth in thyroidectomized rats treated with iodinated Ardein N4 SF*

No. of rats	Amount injected daily mg.	Body weight (g.)		Maintenance %
		Initial	Final	
5	0 (I.C.)*	41	183	100
17	1	43	184	100
3	0 (T.C.)*	44	84	0
8	1	45	172	95

\* I.C. = intact controls; T.C. = thyroidectomized controls.

The daily injection of 1 mg. of this substance into thyroidectomized rats maintained an almost completely normal rate of growth; eight (40 %) of the animals survived for 12 weeks. The injection of a similar quantity of this material into intact rats produced no obvious deleterious effect; seventeen (85 %) survived.

*Restoration of growth with iodinated Ardein N4 SF.* Groups of thyroidectomized rats 42 days after operation, were injected daily for 7 weeks with varying amounts of this preparation. In the first group 1, 2.5 or 5 mg. were injected daily, but as soon as it became obvious that growth was being rapidly restored in these rats the daily amount given to the second group was reduced as shown in Table 3.

Table 3. *Restoration of growth in thyroidectomized rats by iodinated Ardein N4 SF injected daily for 7 weeks*

No. of rats	Body weight (g.)		Amount injected daily mg.	Body weight g. Final	Restoration %
	Initial	When first injected			
12	45	77	0 (r.c.)*	88	0
9	41	137	0 (r.c.)*	218	100
5	43	76	0.001	93	8
4	44	77	0.005	107	27
11	44	69	0.01	101	30
7	43	82	0.02	113	28
11	46	76	0.03	110	33
7	46	76	0.05	125	54
8	44	82	0.10	135	60
8	46	77	0.15	132	63
12	45	70	0.25	154	104
6	44	80	0.50	153	89
15	45	70	1.00	143	89
9	43	76	2.50	165	111
13	43	80	5.00	157	94

\* r.c. = thyroidectomized controls; i.c. = intact controls.

It can be seen that the daily injection of an amount as little as 5  $\mu$ g. produces an appreciable effect on growth; a five-fold increase in this dose, however, causes no further increase in response. The maximum response is not elicited until the daily dose is again raised by ten times to 0.25 mg. Doses up to twenty times this amount produced no evidence of over-stimulation.

Five thyroidectomized rats were similarly injected daily with 2.5 mg. of Ardein (DT/A/630). Increase in body weight during the injection period of 7 weeks amounted to only 14 g., which represents a restoration of growth of only 4%.

*Restoration of growth with iodinated Ardein N4 MB.* Three groups of thyroidectomized rats were injected daily for 7 weeks with varying amounts of iodinated Ardein N4 MB, as shown in Table 4. Complete restoration of growth was achieved by the injection of 0.05 mg. of this preparation. On this basis, therefore, this preparation, containing 0.88% acid-insoluble iodine, is about five times as active as iodinated Ardein N4 SF, having an acid-insoluble iodine content of 0.5%.

Table 4. *Restoration of growth in thyroidectomized rats by iodinated Ardein N4 MB injected daily for 7 weeks*

No. of rats	Body weight (g.)		Amount injected daily mg.	Body weight g. Final	Restoration %
	Initial	When first injected			
3	45	63	0 (r.c.)*	63	0
9	45	129	0 (r.c.)*	180	100
6	46	76	0.002	99	45
4	46	58	0.01	80	43
9	43	64	0.05	112	94

\* r.c. = thyroidectomized controls; i.c. = intact controls.

## RESTORATION OF GROWTH WITH DESICCATED THYROID POWDER

It was shown previously [Rowlands, 1942] that body growth could be restored by the daily injection of 1 mg. of a desiccated thyroid powder (Armour), but no attempt was made to investigate the response in relation to dosage.

On the basis of previous experience, thyroidectomized rats were injected daily with 0.5, 1 or 2.5 mg. of desiccated thyroid (Boots). The results, given in Table 5,

Table 5. *Restoration of growth in thyroidectomized rats by desiccated thyroid powder injected daily for 7 weeks*

No. of rats	Body weight (g.)		Amount injected daily mg.	Body weight g. Final	Restoration %
	Initial	When first injected			
4	43	73	0 (t.c.)*	80	0
9	45	129	0 (i.c.)*	180	100
5	45	71	0.001	95	39
7	46	70	0.005	93	16
6	43	70	0.01	96	43
7	42	68	0.025	97	50
7	42	76	0.05	116	75
10	43	75	0.10	113	71
7	46	72	0.25	118	89
2	40	63	0 (t.c.)	80	0
5	41	118	0 (i.c.)	183	100
5	42	70	0.50	143	117
8	42	74	1.00	129	80
5	46	76	2.50	108	31

\* t.c. = thyroidectomized controls; i.c. = intact controls.

show that restoration was complete, or even that growth might have been accelerated as the result of the administration of 0.5 mg. daily. Over-stimulation resulted from the injection of larger amounts; restoration of growth in rats injected daily with 2.5 mg. amounted to only 30 % of the maximum obtained. As some considerable period of time elapsed between this experiment and those which followed, when much smaller doses of desiccated thyroid were given, the results obtained were calculated from a new set of intact and thyroidectomized control rats, as shown in Table 5. It can be seen that a ten-fold increase in dose from 1  $\mu$ g. to 0.01 mg. gave no significant increase in response. The minimal amount required for complete restoration of growth is between 0.25 and 0.5 mg. daily.

## DISCUSSION

*Restoration of growth in thyroidectomized rats as a test for thyroidal activity*

It has been shown that growth as determined by measurement of body weight can be completely restored in thyroidectomized rats by the administration of desiccated thyroid powder or iodinated Ardein. The outstanding feature of the data given above is the great range of dosage over which preparations of iodinated Ardein and desiccated thyroid are partially effective before the maximal response is elicited; the ratio of the doses required to produce minimal and maximal response is about 1:200. The curve relating dose to response is, therefore, so flat that very large numbers of animals would have to be injected at each level of dosage to make the differences in

response statistically significant. I am indebted to Mr K. L. Smith of the Pharmacological Department, Boots Pure Drug Company, for calculating that an assay comparing a sample against a standard would use 6100 animals if an estimate with an error of 10% at  $p=0.95$  were desired. There are, moreover, other disadvantages. The over-all duration of the experiment is 90 days, of which the first 42 days are necessary for the cessation of growth following the operation. During this time the mortality resulting from deficiency of thyroid secretion may be as high as 60%. The mortality during the actual test period is small, particularly when the amount of substance injected causes moderate or complete restoration of growth and an obvious improvement in the general condition of the test animals. Further, the close attachment of the anterior border of the thyroid gland to the larynx renders complete thyroidectomy in the rat difficult. Consequently, the number of animals available for test may be reduced by the elimination of those whose growth rate after operation suggests that regeneration of some residual fragment of thyroid tissue had occurred. At the end of the injection period the neck region of each animal is inspected and any tissue suspected of being thyroid gland is examined histologically. Confirmation of this may again reduce the number of animals, with the result that finally there remain, on the average, about 30–40% of the number of rats which were thyroidectomized. The use of the growth-restoring action of these preparations for the routine biological assay of thyroidal activity is clearly impracticable.

#### SUMMARY

1. The retardation of growth caused by thyroidectomy in young male rats can be overcome by the implantation of tablets of thyroxine or its mono-sodium or di-sodium salts.
2. No detectable loss in weight of thyroxine or mono-sodium thyroxine tablets occurred in 100 days of implantation. Loss in weight of the tablets of di-sodium thyroxine over a similar period of implantation amounted to about 10%, equal to that of thyroxine tablets implanted for 600 days.
3. Desiccated thyroid powder or iodinated Ardein maintained and restored growth in thyroidectomized rats. The amounts of active material required to produce minimal and maximal responses are in the ratio of 1:200. This and other disadvantages make it impracticable to use the restoration of growth in thyroidectomized rats as a method for the biological assay of thyroidal activity.

The work on iodinated Ardein was undertaken as part of a programme of research on iodinated proteins organized by the Agricultural Research Council. The preparations used were made under the supervision of Dr C. R. Harington, F.R.S., by Mr S. S. Randall of Boots Pure Drug Company. The iodine analyses were carried out by Mrs R. V. Pitt Rivers, who also prepared the di-sodium salt of thyroxine used in the earlier work. Acknowledgement is made to Mr A. Carlyle for assistance in performing thyroidectomy.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 7. USE OF *RANA TEMPORARIA* TADPOLES FOR THE ASSAY OF THYROIDAL ACTIVITY

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(Received 13 October 1944)

The induction of premature metamorphosis in tadpoles by the administration of thyroid preparations, first shown by Gudernatsch [1914], has been studied from various aspects in a number of Anura, most commonly in one or other species of *Rana*. Morse [1914] and Rogoff & Marine [1917] noted the reaction of *R. pipiens* tadpoles to thyroid and other substances, and found that a similar effect could be produced by iodinated blood protein. Lenhart [1915] suggested that the tadpole response could be used for biological assay. Romeis [1923] studied the reaction of tadpoles to thyroxine and di-iodotyrosine in some detail and noted the minimal active doses. Blacher [1928] and Allen [1932], working respectively with tadpoles of *Rana* and of *Bufo*, studied the sequence of response of different organs to thyroxine. Alphonse & Baumann [1935] found that *Rana temporaria* tadpoles with back legs 2 mm. in length responded very rapidly to thyroxine. Kaer [1934] studied the effects on tadpoles of iodinated albumin and other iodine-containing substances and showed that some of the substances had the same effect as thyroxine although much larger quantities were required to evoke a comparable reaction. Gaddum [1927] first tried to use the tadpole reaction as a quantitative measure of thyroidal activity, but his work was incomplete when the *R. temporaria* season came to an end. After considering and rejecting various possibilities of assessing the results quantitatively, Gaddum decided to measure the average decrease in total length caused by different amounts of various substances having thyroidal activity. More recently Wokes [1938] has made a detailed study of the use of *R. temporaria* tadpoles for assay of thyroid powders; his paper is further referred to below. Dutt & Mukerji [1942] carried out thyroxine assays by similar methods on *R. tigrina* and *Bufo melanostictus* in Calcutta and regard the method as satisfactory, apart from the important fact that the tadpoles are limited to a particular season of the year. Their results are expressed graphically like those of Kaer [1934] and Gaddum [1927]. Reineke & Turner [1942] used the tadpoles of *Rana pipiens* for assaying the thyroidal activity of iodinated proteins. Unlike previous workers who had added the substance to be tested to the water in which the tadpoles were kept, these workers injected the tadpoles, which are very large in this species, and they claimed that the injection technique reduces the variability of the results. They used the average percentage decrease in body length as their quantitative index of response and found that it had a linear relation with the logarithm of the dose. The conditions of the test are discussed in some detail and Reineke & Turner conclude that it will distinguish the relatively large differences in activity found in iodinated proteins prepared by

different chemical methods. However, individual variation in response was so large that animals deviating markedly were in some cases omitted from their group averages.

Previous work indicates, therefore, that there are no insuperable obstacles to the use of *Rana* tadpoles for the assay of thyroidal activity, but there are a number of practical difficulties. Firstly, there is the limited season during which tadpoles are available, a difficulty which the expedients referred to below have not overcome so far as *R. temporaria* is concerned. Secondly, tadpoles due to metamorphose 2-3 months after hatching show a steadily increasing sensitivity to thyroidal extracts as they get older and consequently early tests are not directly comparable with later ones. Thirdly, the assessment of the result is laborious. Fourthly, maximal effects tend to be lethal.

The present paper records various experiments on *R. temporaria* tadpoles during two seasons.

#### HATCHING AND REARING OF TADPOLES

##### *Induction of ovulation in Rana*

Considerable difficulty has been experienced in artificially controlling the reproductive processes of *R. temporaria*. Under natural conditions in this country oocytes gradually ripen in the interval between spawning seasons, mainly before the winter, and by March the ovary weighs several grammes and consists largely of a mass of large black ova. Ovulation takes place into the body cavity and the eggs are then collected into the oviducts, which become enormously distended. Finally, oviposition accompanied by mating takes place and the cycle starts again. Bellerby [1933] showed that ovulation could be induced in January or February by a single injection of gonadotrophic extract of anterior pituitary gland, but that oviposition rarely followed, and then only on a very small scale. A single injection had no effect on the ovary earlier in the cycle, though a series of injections increased its size and sometimes led to ovulation. The experience of other workers with *R. temporaria* in this country seems to have been similar [Waring, Landgrebe & Neill, 1941]. On the Continent, however, several authors, including Rostand [1935], Ponce [1936] and Gallien [1937], have recorded that ovulation and oviposition of fertilizable eggs can be induced in *R. temporaria* any time during the winter from November onwards by the injection of pituitary preparations. This result is possibly associated with the observation that on the Continent the ovary of *R. temporaria* has reached nearly maximum size by the autumn [Rostand, 1935].

Experiments with other species of *Rana* and with *Bufo* have been more promising [Rugh, 1935]. *Rana pipiens*, when it is available, is suitable for assay purposes since tadpoles can be obtained during the autumn and winter if the female is injected with macerated pituitary glands of the same species and a sperm suspension is added to the eggs [Rugh, 1934]. To produce sufficient tadpoles out of season for routine testing on a large scale is laborious, since for a batch of fertilized eggs three or four adults must be killed [Rugh, 1934]. Reineke & Turner [1942], however, have successfully used the species. A recent report by Kehl [1944] indicates that *Discoglossus pictus* may be a useful species, since it has a long breeding season in nature, and if kept in good condition will lay eggs in captivity at almost any time of year.

*Source and management of tadpoles*

Some workers with *R. temporaria* seem to have relied on odd groups of tadpoles obtained in the swimming state from ponds. It is better, however, to obtain masses of newly deposited spawn from which hatching will take place in the laboratory. The natural spawning season in any particular locality varies from year to year according to environmental conditions which are not fully understood [Savage, 1935*a*]. From the environs of North London we obtained almost unlimited spawn between 15 March and 7 April 1944. No doubt supplies of spawn could be obtained much earlier from the south-west and much later from the north, so that the season for laboratory work could be extended by collection of spawn from a wide area. Since *R. temporaria* lays its eggs in a solid mass, a thousand or more 'litter mates' can be hatched in the laboratory for assay work. The youngest eggs we have obtained were fully segmented, but at ordinary room temperature (about 20°C.) about 3 days elapse before they begin to elongate. In another 2 days the larvae begin to eat their way out of the gelatinous envelope. For another day or so they hang up on the jelly mass which does not provide appreciable quantities of food, but which has some beneficial effect on development [Savage, 1937]. The tadpoles then become free-swimming and feed voraciously off small pieces of liver or other meat. Hatching is most conveniently carried out in large Petri dishes, so that as soon as the tadpoles become free-swimming they can be pipetted out into clean water in other containers. No trouble has been experienced in the use of London tap water.

Systematic experiments on density and food intake were not carried out, but 10-20 tadpoles per litre make excellent progress when fed on liver, provided the water is changed every second day. This is necessary not only because *Rana* tadpoles feeding on fresh meat rapidly foul the water, but also because of their comparatively high oxygen requirement. Experiments with *Rana* tadpoles in boiled and unboiled water in sealed and unsealed flasks showed that their oxygen requirement was much greater than that of *Xenopus* tadpoles. When the water is lacking in oxygen, the tadpoles swim upwards, even at a stage of development when they would otherwise be motionless [Savage, 1935*b*]. When it is very stale the tadpoles float on the surface where the oxygen supply is least inadequate and show little activity. The shape of the container had no perceptible effect on growth. Under good conditions of temperature, density and food supply some tadpoles reach 30 mm., the size at which we have used them for assay, in about 3 weeks. Growth is much retarded by adverse conditions, such as crowding.

*Retardation of development*

Unless special measures are taken to retard development, most *Rana* tadpoles kept in the laboratory have metamorphosed or are too advanced for assay purposes by the end of June. The fact that the rate of development of anuran eggs and tadpoles is greatly influenced by temperature suggests that the season could be extended for assay purposes by maintaining the eggs and afterwards the tadpoles at a temperature at which development is slow. Hertwig [1898] found that gastrulation in *R. temporaria* took place in a little over 1 day at 20°C., in about 3 days at 10°C., and not before 3 weeks at 1°C. At the lower temperature the formation of the neural plate took

more than a month. We carried out an experiment to determine how long and under what conditions this slow rate of development could be maintained.

Two large batches of spawn were put into two separate jars in about 2 l. of water, and put in cold store at about  $1^{\circ}\text{C}$ . A sample of each lot was removed immediately as a control and allowed to hatch at room temperature. The sample of batch 1 produced 58 tadpoles; 38 eggs did not hatch. Batch 2 sample produced 65 tadpoles; only 2 eggs did not hatch. Further samples were removed from both batches at weekly intervals and allowed to hatch at room temperature, the water in the containers in the cold store being changed each 2 weeks. The hatches of the samples were very good up to 4 weeks in cold storage, the spawn showing no deterioration. It was evident, however, that slight development was proceeding in the cold store, and at 5 weeks some eggs were beginning to elongate. Good hatches, however, were still obtained from eggs brought to warm surroundings. At 6 weeks the spawn looked much less healthy and only poor hatches were obtained from samples. At 7 weeks much of the remaining spawn was decomposing; the sample from one batch failed to produce any tadpoles, that from the other produced a few only. It is probable that more frequent changing of the water in the cold store would have prolonged the life of the eggs, but the spawning season had ended before further experiments could be undertaken. Even so, it is obvious that the development of the eggs can be delayed for at least 5 weeks by storing at slightly above freezing-point. Attempts to arrest completely the development of the eggs for a time were made by freezing the spawn at  $-1$ ,  $-10$  or  $-75^{\circ}\text{C}$ . At the highest of these temperatures freezing was slow, possibly owing to supercooling. Once the spawn had been frozen solid no hatching could be obtained on warming, but hatches were obtained from eggs subjected to  $-1^{\circ}\text{C}$ . for some days in cases where ice formation was not complete. Many of the eggs thawed out of solid ice were obviously ruptured. At  $-10^{\circ}\text{C}$ . ice formation was, of course, rapid and death of the eggs, with rupturing, was invariable. Eggs frozen at  $-75^{\circ}\text{C}$ . (solid  $\text{CO}_2$  in alcohol) in water were ruptured but not those frozen without surrounding water. No hatches were obtained from eggs so treated, except in one case where the eggs were exposed to dry  $\text{CO}_2$  snow for a very short time, and there was some doubt as to whether freezing was complete. Partial dehydration of the eggs in *M* sucrose for 24 hr. was itself lethal; short periods did not increase resistance to freezing. These results are quite in keeping with the current theory that living tissue can only survive low temperatures if cooling is sufficiently rapid to produce instantaneous vitrification, without the formation of ice crystals [Luyet & Gehenio, 1940]. Such rapid cooling could obviously not be achieved at  $-75^{\circ}\text{C}$ . with as large a body as a frog egg.

Experiments were also carried out on retardation of development at other stages of development. Two lots of larvae just before hatching were transferred from  $20$  to  $1^{\circ}\text{C}$ . for 3 days without adverse effect, though development was arrested for the 3 days in cold store. Tadpoles about a week after hatching were also found to survive sudden transference to  $1^{\circ}\text{C}$ ., though apparently they did not feed at this temperature, and they showed considerable mortality in the course of a week. It seems likely that their development at this temperature would be very slow, but storage under these conditions on any large scale would require considerable facilities and much preliminary work under difficult conditions.

Wokes [1938] found that development was much retarded at 8°C. and considered that storage at this temperature would extend the season to September. Some of the spawn from our 1944 collection, housed outside at a temperature varying between 6 and 10°C. developed very slowly and took more than a week to begin to elongate and about 2 weeks to hatch. The weather, however, became warmer and development accelerated. We had no facilities for the maintenance of a temperature of 8°C. Our present experience suggests that severe retardation should not be attempted, since, if feeding is inhibited, the tadpoles will inevitably get into bad condition. Probably the temperature used by Wokes, applied from the early segmentation stages, would, as he suggests, be effective in making tadpoles available right through the summer without serious mortality. Storage at this temperature, however, would certainly involve frequent changes of water, and large-scale operations would present serious difficulties of accommodation.

Retardation of development might also be effected by limitation of food supply, but we have not yet had opportunity to investigate this idea. Maintenance in iodine-free water, according to the observations of Metcalf & Creaser [1937] might also be effective, though it would be difficult on a large scale. Any method of retardation might, of course, greatly affect the response of the tadpoles to thyroidal activity, and where testing facilities are required all the year round it seems preferable to utilize, if possible, one of the species in which oviposition and fertilization can be achieved at any time.

#### ASSESSMENT OF RESPONSE TO THYROIDAL ACTIVITY

The changes which constitute metamorphosis of the tadpoles of *Rana temporaria* are well known and consist, briefly, of gradual growth of the hind legs, slight change in the shape of the head, eruption of the front legs, and shrinkage of the tail. The administration to tadpoles of preparations with thyroidal activity causes these changes to appear prematurely. Whether or not the animal survives the premature appearance of the changes depends on its age, on the rapidity with which the changes are induced, and on the experimental conditions.

In theory, any of the changes of premature metamorphosis could be used for the assessment of the response to thyroidal preparations. In practice, there is some difficulty in putting the response on a quantitative basis. Measurement of the growth of the back legs is not easy; weighing of the whole tadpole to determine the loss of weight due to wastage of the body and shrinkage of the tail presents special difficulties [Gaddum, 1927]. Previous workers using *Rana* had suggested that the appearance of the front legs might be used as a criterion of thyroid activity [Romeis, 1923], but owing both to the high mortality and to the difficulty of detecting the first appearance of the limb [Gaddum, 1927] no satisfactory test along these lines has been worked out. According to Blacher [1928], working with various *Anura*, the eruption of the forelimbs comes last in the order of response of different organs of the tadpole to thyroid (or thyroxine), and the dose used cannot be minimal. This alone would increase the possibility of mortality during the test, but in any case in *Rana* the arm emerges by breaking through the wall of the branchial chamber on the right side and by passing through the spiracle on the left side, blocking up this passage completely; the appearance of arms, therefore, means the abrupt cessation of branchial respiration. If the stimulation has been intense this happens in the

test tadpole before the lungs are functional, and death occurs. Moreover, although front limbs appeared in several of the experiments recorded below, the proportion of tadpoles in which this took place was very variable in relation to the dose and the reaction seemed to be an unsatisfactory end-point for assay purposes. It was decided, therefore, that for a short-term test, it was undesirable to use the front legs as indicators. There remained measurement of total length, which is greatly decreased by the shrinkage of the tail and to a lesser extent by wastage of the body, and, like most previous workers, we adopted this method. Our measurements have been made in a small Petri dish over squared paper, on tadpoles lightly anaesthetized where necessary in 1/5000 chloretone. This technique permits of measurement to be made to about 0.5 mm.

#### METHODS OF TEST ON *RANA* TADPOLES

Owing to the restricted season for *Rana* tadpoles it has not been possible to make a comprehensive examination of even the more obvious variables pertaining to the test, but several different procedures have been tried.

#### *Method of administration*

Preliminary experiments with the tadpoles of *R. temporaria* in 1943 showed that, contrary to the experience of Reineke & Turner [1942] there was no difficulty in inducing shortening and metamorphic changes of the kind described by earlier workers if active preparations of iodinated proteins were merely added to the water as suspensions, and graduated results proportional to the dosage could be obtained. In one experiment, the tadpoles responded more strongly to an alkaline solution of iodinated casein than to the aqueous suspension. However, a preparation with low activity was not more effective after peptic digestion, showing that its activity was not affected by the nature of the suspension. The routine method of administration has accordingly been in fine aqueous suspension.

#### *Time-dose response relations with continuous dosage*

In the early experiments the tadpoles under test were fed on liver and the water was changed daily and a fresh dose of suspension was added. At room temperature the reaction proceeded slowly and the progressive decrease in length of the animals could be plotted. Five tadpoles were kept in a 250 ml. beaker, no attempt being made to identify individuals. Fig. 1 shows graphically the type of reaction produced. Comparatively few tadpoles were available in 1943, but preliminary results made it possible to compare the activity of a number of different preparations. The test was somewhat laborious, since it involved repeated individual measurements of groups of ten tadpoles. Although the sensitivity of the tadpoles increased as the time of normal metamorphosis approached, similar groups could be chosen for the comparison of different doses or different substances. The average decreases in length of 33-34 mm. tadpoles varied from 2 to 14 mm., and were of the same order as those obtained by Reineke & Turner in *R. pipiens* tadpoles of similar size. As a rough method of examining the activity of different iodinated caseins the doses of the various substances required to produce the same decrease in average tadpole length in a single test were compared.

*Dose-response relations with one-day dosage*

In 1944 work was resumed on the *Rana* test and an attempt was made to simplify and shorten it. It was hoped to avoid repeated length measurements since this method tended to limit the number of animals which could be used at any one time. Tests were carried out as before almost entirely with large tadpoles. Five of these were kept in each beaker, the dose of iodinated casein or the substance to be tested was added as a suspension, and the water was made up to 200 ml. Tests were run at higher temperatures than in the preceding year in order to speed up the reaction,

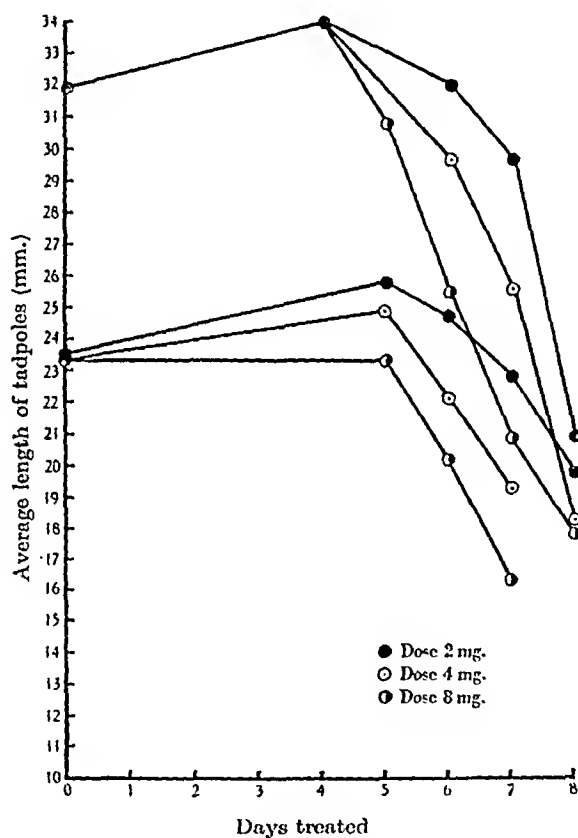


FIG. 1. Average length of groups of five tadpoles treated daily with various doses of iodinated casein, NC3. Curves are shown for two initial sizes.

and each beaker received only a single dose of the active substance. Preliminary tests showed that at beaker temperatures of 24.5–28°C. active iodinated proteins would produce marked changes in the tadpoles after 3 or even 2 days, tap water being substituted for the water containing the suspension under test after the first day. By the fourth day a good many of the tadpoles receiving the larger doses would be dead so that there was nothing to gain by prolonging the test. Control tadpoles subjected to the same temperature without food lost on an average about 0.5 mm. in length.

The majority of tests carried out were therefore of this type and the decrease in length on the third day showed a fairly consistent relation to the dose. Tadpoles of

28-33 mm. were used in almost all tests although smaller ones were found to be nearly as sensitive; the advantage of large ones was that they provided a bigger margin for decrease in length.

It was found that the range of dosage of the iodinated casein preparation NC4+5 for *Rana* was of the same order as that used for *Xenopus*, 0.35-2.0 mg. per 200 ml. beaker containing five tadpoles. The doses used in 1944 were lower than in 1943, since the effects were intensified by the higher temperatures. These could not always be stabilized and temperature changes accounted for some of the variability of the results.

In the first series of tests the tadpoles were graded for size at the beginning of the tests but not individually measured; it was thought that the decreases which would be produced experimentally would be sufficiently great for detailed initial measurements to be unnecessary. A number of tests were carried out on these lines and where only preliminary results as to the activity of a substance or its optimum dose level were required the method was reasonably satisfactory. Table 1 shows

Table 1. *Response of Rana tadpoles to 1-day dosage with iodinated casein (NC4+5). Tadpoles not individually measured at the beginning of the experiment*

Test no. ... ..	8	9	10	
Range of initial length ...	28-27 mm.	31-29 mm.	33-30 mm.	
Dose (mg. per beaker)	Average length after 2 days	Average length after 2 days	Average length after 3 days	
0.25	—	—	28.2	28.7
0.5	25.5	—	27.3	28.7
1.0	24.4	—	25.8	25.6
2.0	20.2	22.5	—	—
4.0	—	21.0	—	—
8.0	—	16.9	—	—

typical results obtained on similar tadpoles between 6 and 10 April at different temperatures. In test 8 the temperature was high (almost 28°C.) and in test 9 the dosage was high; these tadpoles showed appreciable shrinkage after 2 days, but a high mortality. Further experiments were carried out to see if the decrease to 25 mm. or less could be taken as the end-point to the reaction in an individual tadpole, but the percentage of animals decreasing to this size did not satisfactorily correspond to different dose levels. It was finally concluded that tadpoles must be measured individually in order to distinguish a graduated response to graduated doses of iodinated caseins or other thyroid-active substances. In the present work tadpoles were always kept five in a beaker (partly owing to a shortage of incubator space), but the statistical value of the results would have been greater if each tadpole had been kept separately so that individual decreases could have been recorded. Individual variability appears to be very high as previous workers have found, and there is some evidence that it increases with age. Table 2 and Fig. 2 show the results of five dose-response tests on *Rana* carried out at approximately the same temperatures in which 220 tadpoles were used and individually measured at the beginning and end of the 3-day experiment. It will be seen that the results, though variable, show a reasonable correspondence with the dosage. In a further test (45) 0.7 mg. of the standard (NC4+5) was given to two sets of forty tadpoles in sixteen



beakers of five each; the average decrease per beaker at the higher temperature was 6.5 mm. and at the lower temperature 3.4 mm., but it varied from 3.8 to 9.7 mm. and from 2.0 to 6.7 mm.

Table 2. *Response of Rana tadpoles to 1-day dosage with iodinated casein (NC4+5). Response measured as decrease in average length at end of third day*

Test no. ... ..	20	26	27	30	31
Average initial length ...	27.7 mm.	31.2 mm.	31 mm.	29.5 mm.	30.1 mm.
Dose (mg. per beaker)					
0.35	—	—	1.4	2.6	2.0
0.5	—	3.3	2.3	2.9	2.6
0.7	3.7	—	2.3	5.9	5.5
1.0	4.2	4.5	6.3	5.3	7.8
1.4	6.4	—	6.0	6.2	8.3
2.0	—	10.4	—	—	—
4.0	—	11.1	—	—	—

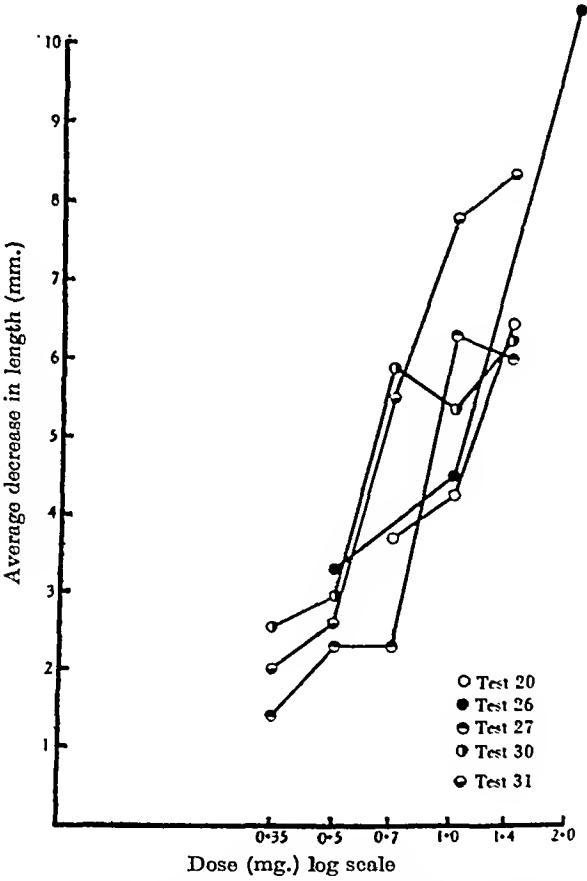


FIG. 2. Average decrease in length after 3 days in groups of ten tadpoles receiving, for 1 day, various doses of iodinated casein NC4+5. Five separate tests.

At temperatures of 25°C. and above, the dose-response curve is fairly steep though it tends to be flat at the beginning and end.

Comparison of an unknown preparation with the laboratory standard (NC4+5) was carried out by doing a simultaneous test on two or three different doses of each

[Deanesly & Parkes, 1945]. A test carried out at the beginning of June suggested that tadpoles retarded in their early stages responded less uniformly and less vigorously than those which had developed steadily, but satisfactory tests were still carried out up to the middle of July.

*Residual activity of suspensions*

Experiment showed that with beaker doses of 0.7, 1.0 and 1.4 mg. of NC4+5 the reaction of the tadpoles was not appreciably greater if the dose was kept in the water for 2 days instead of 1 day. The average decreases on the third day for doses of 0.7, 1.0 and 1.4 mg. respectively were 4.0, 5.5 and 7.5 mm. in the groups dosed for 1 day, and 3.4, 4.5 and 8.6 mm. in the groups dosed for 2 days. This agrees with the findings in another test in which the water containing the iodinated casein was tested for residual activity after tadpoles had been in it for 24 hr. For doses of 1, 2 and 4 mg. of NC4+5 in this test (22) the average decreases in length in the original groups were respectively 1.8, 2.1 and 4.2 mm.; the residues from the first two doses produced no significant decrease in length in fresh groups of tadpoles and the average decrease in length caused by the residue of the 4 mg. dose was 1.3 mm., less than that caused by 1 mg. of NC4+5. This test indicates that all or most of the activity disappears from the beakers after 24 hr. with doses up to 2 mg., but slight residual activity persists from higher original doses. In a similar test (23) carried out at a higher temperature, 1 mg. of NC4+5 caused a decrease of 5.3 mm. in the first group of tadpoles but only of 1.8 mm. in the tadpoles exposed to the residue of the dose. It is clear from these experiments that *Rana* tadpoles remove the active material from the medium much faster than did *Xenopus* tadpoles in the experiments described by Deanesly & Parkes [1945].

*Relative effect of concentration and absolute amount of the dose*

An experiment was carried out by the continuous dosage technique to see if the response of the tadpoles to iodinated casein was affected by the volume of water in which the dose was placed. Four tadpoles were put in 165 ml. in beaker A, and four similar ones in 1650 ml. in beaker B. Each beaker received 3 mg. of NC2 daily. After 6 days the tadpoles in beaker A had decreased 10.6 mm. in length and those in beaker B only 3.4 mm., showing that the tadpoles had taken up less of the active substance from the weaker suspension.

Table 3. *Relative effect of concentration and dose per tadpole*

Volume of medium ml.	Dose mg.	No. of tadpoles	Concentration mg. per l.	Amount per tadpole mg.	Decrease in length mm.
200	1.0	5	5.0	0.2	3.3
2000	10.0	20	5.0	0.5	4.0
2000	10.0	10	5.0	1.0	7.7
400	0.5	5	1.25	0.1	1.9
200	0.5	5	2.5	0.1	2.2
400	1.0	10	2.5	0.1	1.9
2000	4.0	10	2.0	0.4	5.6
400	2.0	5	5.0	0.4	7.8
200	2.0	5	10.0	0.4	6.3
2000	20.0	10	10.0	0.5	8.4
400	4.0	5	10.0	0.8	8.9

Later experiments (Table 3) with the 1-day dosage technique showed that with a constant amount per tadpole the more concentrated solutions gave the best results, but with a standard concentration increasing the amount per tadpole increased the response.

#### CONCLUSIONS

A comparatively large number of tests were carried out, but many were preliminary in character, and mathematical treatment of the results has not been attempted. Our experience agrees with that of previous workers in showing that the test can give valuable indications of the thyroidal activity in a preparation, but that it is quantitatively unsatisfactory.

Tadpoles should be of the same age and size and kept under the same conditions for any one test, and unknown preparations should be tested against a standard substance. It is desirable to maintain the tadpoles at a constant temperature during the test.

If rearing is controlled tadpoles can be used for testing from April till July, but a further extension of the period would probably not be worth the labour entailed.

The response most easily measured is the decrease in total length, so that tadpoles must be kept singly if the full extent of individual variability is to be ascertained, and to obtain statistically significant results on a large number of preparations would require more animals than could easily be kept under suitable conditions in the time available.

#### SUMMARY

1. The use of tadpoles for the assay of thyroidal activity is discussed with special reference to *Rana temporaria*.

2. The restricted season for *Rana* tadpoles makes it difficult to carry out a detailed examination of variables, but the following have been considered: method of administration, duration of dosage, concentration and absolute amount of dose, method of assessing result.

3. For a short test tadpoles were kept at 25–27°C., dosed for 1 day, and measured at the beginning of the experiment and at the end of the third day.

We are much indebted to R. Maxwell Savage, Esq., F.I.C., F.Z.S., for assistance in obtaining spawn. The thanks of one of us (J. E.) are due to the Directors of Boots Pure Drug Co. Ltd. for arranging facilities for participation in this work.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 8. USE OF *XENOPUS* TADPOLES FOR THE ASSAY OF THYROIDAL ACTIVITY

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(Received 13 October 1944)

### REARING OF *XENOPUS* TADPOLES UNDER LABORATORY CONDITIONS

#### *Introduction*

*Xenopus laevis*, the clawed toad, a native of South and West Africa, has long been known in this country, and it has become of exceptional interest as an anuran which can easily be bred and reared under laboratory conditions. Bles [1905], who summarized a few earlier papers on the species, gave an account of its life history and breeding habits based on animals kept in captivity for 7 years. Bles kept the toads, which are purely aquatic, in a tropical aquarium at about 25°C. in summer (occasionally 28–30°C.); they were fed daily with small earthworms or thin strips of calves' liver. The water in the aquarium was never changed, but the bottom was covered with earth and stones and *Vallisneria* thrived in it. In December the temperature was allowed to sink to 15–16°C. during the day and lower at night. The animals became sluggish, took little or no food, and practically hibernated. Bles found that he could stimulate pairing in spring by raising the temperature of the aquarium to 22°C. and then running in cool spray to imitate the fall of rain. From one female alone between April and July he obtained more than 15,000 eggs. Spawning took place during February, April and May in successive years and in one year during May, June and August. In South Africa spawning normally occurs between July and September, i.e. midwinter and early spring [Shapiro & Shapiro, 1934; Berk, 1938]. Bles described the segmentation of the egg, the development and hatching of the tadpole, and its early free-living stages. Later workers have confirmed Bles's results in general, including Koffhaus [1933], Holmgreen [quoted by Zondek, 1935] and Vanderplank [quoted by Berk, 1938]. The essential feature of these successful attempts to induce oviposition and mating appears to have been change of environmental conditions.

Hogben introduced *Xenopus* into this country from South Africa in considerable numbers for physiological work. Reports of an adverse effect of captivity on the ovaries [Zwarenstein & Shapiro, 1933; Shapiro & Shapiro, 1934] were not substantiated; it was soon found that toads kept at a suitable temperature and with adequate feeding would remain in a healthy state for an indefinite period, and optimal conditions were worked out in some detail [Alexander & Bellerby, 1938; Bellerby, 1938; Landgrebe, 1939]. In a constant laboratory environment oviposition never occurs spontaneously, but the ovary remains fully developed and is highly sensitive to

gonadotrophic substances. The injection into the dorsal lymph sac of a suitable amount of gonadotrophin from almost any source (e.g. 200 i.u. chorionic gonadotrophin) causes discharge of ripe ova [Hogben, 1930; Bellerby, 1933; Weisman & Coates, 1943]. In this species, unlike *Rana*, ovulation is followed immediately by oviposition, the eggs being carried from the body cavity, down the oviduct and thence to the exterior by continuous ciliary motion [Waring, Landgrebe & Neill, 1941]. *Xenopus* is, in fact, the only laboratory animal which shows an externally visible sign of gonadotrophic stimulation and which can be used repeatedly for test purposes. For this reason it has been used extensively for pregnancy diagnosis [Bellerby, 1934; Shapiro & Zwarenstein, 1935; Landgrebe, 1939; Landgrebe & Samson, 1944], and it was introduced into the United States for this purpose from Prof. Crew's Pregnancy Diagnosis Laboratory [Weisman & Coates, 1941]. If the male is injected simultaneously, or sometimes even if untreated, coupling takes place, the eggs are fertilized, and viable tadpoles are produced [Shapiro, 1936].

Most of the work carried out on *Xenopus* in the last few years has been concerned with the adult, but our interest has centred mainly on the rearing of tadpoles for assay purposes, and has involved a study of optimal conditions of maintenance, feeding, etc. Bles describes the breathing and feeding mechanism of the tadpole. The newly hatched tadpole has transitory external gills, but by 6 days after hatching the operculum has grown back and fused in the mid-ventral region leaving only the spiracles open. At this time a branchial current of water has been set up through the mouth, but the alimentary canal still contains yolk. Bles states that within 2 hr. after the first appearance of faeces the tadpoles rise to the surface for air and begin to use their lungs as breathing organs. Beddard [1894] had thought that respiration was carried on in the branchial arch region although no true internal gills were developed, but Bles maintains that the lungs are not only hydrostatic but respiratory in view of the frequency with which the tadpoles rise to the surface.

Bles agrees with older writers in finding that *Xenopus* tadpoles lack the horny teeth found in other species. They feed by sieving out the small particles and organisms from the water which is gulped in and passes out through the spiracles. Bles believed that this current of water had no respiratory function. These early conclusions about the feeding mechanism have been fully substantiated by the general experience of later workers, who found that, unlike *Rana* tadpoles, *Xenopus* tadpoles are unable to live on pieces of meat [Shapiro, 1936] but thrive well in pond water [Landgrebe & Purser, 1941] or on *Chlamydomonas* cultures in hay infusion [Landgrebe & Samson, 1944]. Fine suspensions of raw liver [Elkan, 1939] or of powdered dried nettles or clover [Gasche, 1943] are also effective nutrients.

The tadpoles are extremely sensitive to temperature and do best at about 25°C. [Elkan, 1939]. According to Gasche, metamorphosis of the best-grown tadpoles of a batch starts 5-7 weeks after oviposition, and the toads reach sexual maturity in 5-6 months (males) or 7-8 months (females). Landgrebe & Samson found that metamorphosis began at about 10 weeks, and record that a female toad fed on live *Lebistes* attained a weight of 25 g. in 8 months. They also obtained ovulation by the injection of chorionic gonadotrophin in females of only 16 g.

Factors affecting the growth of *Xenopus* tadpoles (other than temperature and food) do not seem to have been investigated to any large extent. Some information

regarding size and shape of container, renewal of water, density of tadpoles, etc., is available, however, from other species. It has been shown, mainly on *Rana* tadpoles, that the growth rate depends largely on the volume of water available per individual; this is true for other aquatic individuals, and Pflüger [1883] suggested that mechanical disturbance was a hindrance to growth. Bilski [1921], working with *Bufo* and with *Rana esculenta*, found that frequent changes of water or crowding would retard tadpole growth. He attributed this retardation to the increase in the number of contacts and found that to increase the growth rate it was better to reduce the number of tadpoles in a given vessel rather than to increase the volume of water. Adolph [1931] obtained similar results with *R. pipiens* and *R. sylvatica*. Sixty-four, thirty-two, sixteen and one tadpoles were put respectively into four similar dishes each containing 500 ml. of water and ample food; after 4 weeks the most crowded tadpoles averaged one-twelfth of the weight of the one alone. Experiments showed that nothing added to or subtracted from the water by other tadpoles was responsible for the effect of crowding, and within the limits tested the tadpoles were not affected by the surface area of the water. The effect of crowding is not visual since it is shown in the dark. In agreement with other observers, Adolph noted that tadpoles ate less in crowded colonies, a conclusion also arrived at for adults by Bellerby [1938]; Adolph also found that overcrowding of tadpoles led to individual diversity in weight much greater than when growth was under optimal conditions. Rugh [1934] carried out further experiments along these lines under carefully controlled conditions, and Lynn & Edelmann [1936] studied the relation of crowding to the time of metamorphosis. Adolph [1931] had already shown that uncrowded individuals tended to metamorphose earlier, and this was confirmed by Lynn & Edelmann. These authors accept the view that the retardation in crowded cultures is associated with the increased movement due to frequent contacts. In view of these results it seemed important to ascertain the optimum conditions for rearing *Xenopus* tadpoles.

#### *Oviposition and hatching*

We have little to add to the information recorded in the literature on the artificial induction of oviposition. Our toads have been kept at 20–25°C. in a few inches of water in small tanks, and fed heavily three or four times a week on strips of fresh rat or rabbit liver. The injection of 200 i.u. of chorionic gonadotrophin has usually been adequate to cause oviposition, though 500 i.u. have occasionally been necessary. The males have received 100 i.u., and coupling has occurred almost invariably. Landgrebe & Samson [1944] recommend that the toads should be mated on a metal grid through which the eggs fall so that they cannot be eaten. We have achieved the same effect by inverting a large Petri dish in the bottom of a filtrate jar, leaving round the sides about  $\frac{1}{4}$  in. gap down which the eggs fall. It was thought that this all-glass set-up might be safer than metal grids. As a routine we have made the injections at midday, coupling and oviposition then start during the evening and are usually completed by the morning. The females have been used at intervals of about 6 weeks, and some twenty batches of tadpoles have been reared from three females in the course of a year. On one or two occasions the whole batch of eggs has been infertile even where coupling has occurred. By contrast, a high percentage of fertile eggs with up to 2000 tadpoles counted out in the free-swimming stage have

been secured in other batches. Much larger numbers have, however, been obtained by other workers.

Good eggs can be distinguished at oviposition by their having a definite black hemisphere—the animal pole—which floats uppermost and which, if fertilization has taken place, rapidly spreads over the rest of the egg, so that a uniform brown coloration is produced. Eggs of a blotchy grey colour are infertile. The eggs are pipetted into small dishes in such numbers that they are thinly scattered on the bottom. The rate of development depends largely on the temperature, but is remarkably rapid. At 25°C. they elongate in about 24 hr. and hatching is completed a day later. Shortly afterwards the larvae rise to the surface and hang from the surface film or from the side of the vessel, sometimes collected in small bunches, sometimes singly. By this time, if any large proportion of infertile eggs are present, the water will be getting foul, and the newly hatched larvae are pipetted into clean water. About 24 hr. afterwards the tadpoles are beginning to swim freely; later the mouth opens and the animals begin to feed. They become continuously free-swimming about 4 days after oviposition, and are then transferred to tanks or large jars at the same temperature.

### *Rearing tadpoles*

In the course of experiments on the rearing of *Xenopus* tadpoles the following variables were examined—temperature, feeding, number per litre (density), aeration, size and shape of container, and changing of water. Unless otherwise mentioned, the tadpoles were kept in 10–12 l. tanks or in 2–3 l. jars. At first, gravel, pond weed, snails, etc., were introduced with the idea that the simulation of natural conditions would assist, but such devices were found to be quite unnecessary, and were discarded.

*Assessment of growth.* In all the experiments growth has been assessed by measurements of total length. This system is not ideal. First, length measurements are not easy in *Xenopus* tadpoles, owing to their tails being curved and semi-transparent. Secondly, determination of the weight of the tadpoles would provide a much better indication of change in size. However, attempts to weigh *Rana* tadpoles by previous workers have not been very successful, mainly owing to the difficulty of removing adherent water, a difficulty which persists even when weight is determined by displacement of water in a burette. In the case of *Xenopus*, removal of adherent water is complicated by the fact that the tadpoles will not survive excessive handling. We are not quite in full agreement with Landgrebe & Samson as to the extreme delicacy of *Xenopus* tadpoles, but it is certain that manipulations such as drying off on filter paper and inserting into a burette are very apt to be lethal or retarding, and are therefore quite unsuitable for a growth experiment. Even if only one determination is made (at the end of the experiment) and a high mortality after weighing is of no consequence, the difficulty of standardizing residual water remains.

Length measurements have been made by putting the tadpoles into about 1 cm. of water in a small Petri dish over squared paper. When the tadpole takes up a suitable position its length can be read off within about 0.5 mm. The amount of handling required for this operation rarely affects the tadpoles adversely. In using increase of length as an indication of growth it must be remembered that an increase of 5 mm. from say 20–25 mm. in length, represents a considerable increase in size of the tadpole. We have not determined the relation between length and weight with



any accuracy, but a few estimations of displacement made by the burette method gave results compatible with the assumption that size would vary as the cube of the length. Thus, an increase from 10 to 20 mm. in length represents an increase of eight times in size, and an increase in length from 20 to 30 mm. an increase of more than three times in size.

*Temperature.* Our observations agree with those of previous authors. The tadpoles grow well at 20–25°C. On a large scale, individual warming of small containers was not possible, but the use of an electrically heated room made it possible to maintain a suitable temperature. Below 20°C. growth is slow, at 15°C. it practically stops. Temperatures between 25 and 30°C. are not deleterious over short periods, but as we were unable to maintain such a temperature for rearing generally no information was obtained as to its effect on the growth rate.

*Changing of water.* For large-scale work in London, carried on all the year round, it was impossible to rely on pond water. Tap water, however, was found to be quite adequate provided certain precautions were taken. It was found that, on some occasions, whole batches of tadpoles died after being put into fresh tap water drawn from iron pipes. Information from Dr Landgrebe led us to think that residual chlorine in the water might be responsible for the deaths. Analyses carried out by the Metropolitan Water Board a little later showed considerable day-to-day variation in the chlorine content, though the amount was always small, 0.20–0.01 p.p.m. It seemed unlikely that such amounts could be lethal, but as a precaution the routine was established of adding liver powder (see p. 330) to the tap water (about 100 mg. per gallon) a day or more before use, to absorb any free chlorine. This procedure was highly effective, and mortality among the tadpoles became negligible after its introduction.

The frequency with which the water needs to be changed in an aquarium which is not artificially aerated and does not contain a balanced flora and fauna depends on the need for renewing the oxygen supply and for removing the products of excretion and of the decomposition of excess food. It has been shown by several workers that the removal of excretion products is not important for tadpoles, and fouling of the water by excess food can easily be avoided, in the case of *Xenopus* tadpoles, by adjusting supply to consumption. Moreover, *Xenopus* tadpoles, except when disturbed, are not very active and their oxygen requirement is probably very slight (see section on aeration) and may be partly satisfied by their habit of gulping atmospheric air. These factors together probably account for the fact that *Xenopus* tadpoles, unlike *Rana*, do not require frequent changes of water. In practice we have found it quite satisfactory to leave them unchanged until the majority attain the length of 20–25 mm. (when about 3–4 weeks old) at which size they are used for assay work. Outstandingly large and vigorous tadpoles are not used for assay, but are put aside in fresh containers and allowed to proceed to metamorphosis without further change of water. The stragglers found in most batches are discarded.

*Aeration.* Aeration of the water is usually thought to be an important factor in the success of aquaria, but Bles's account of the respiratory physiology of *Xenopus* tadpoles, our observations on dispensing with changing the water, and the curious results with shape of container, all seemed to throw doubt on its necessity for *Xenopus* tadpoles. Moreover, *Xenopus* tadpoles frequently rise to the surface and

gulp air. To throw further light on the problem, the following experiments were carried out.

A tadpole 1 week after hatching, when the operculum had grown over the external gills, was placed in a 200 ml. beaker and watched for 1 hr. 40 min. During that time it surfaced only six times at fairly regular intervals. Larger tadpoles (28 mm. and just before metamorphosis) surfaced only three times in about the same period. Since the younger tadpoles would be actively growing at the stage observed and the tadpole near metamorphosis might be expected to have well-developed lungs, it is surprising, in view of Bles's conclusions, that neither type of tadpole surfaced more often, and these observations threw doubt on his statement that the tadpoles are primarily air-breathers. Further observations at different times of the day and at different temperatures indicated that small ones might surface as often as nine times in half an hour and large ones as infrequently as once in an hour. Tests were carried out comparing the frequency of surfacing of small tadpoles put into beakers of boiled and unboiled water. The boiled water was covered with paraffin wax during cooling so that it should contain little oxygen when the wax was removed and the tadpole put in. From 5.30 to 6 p.m., at a temperature of 74°F., the tadpole in boiled water surfaced eight times and that in unboiled water only once; the two tadpoles were then exchanged and the tadpole in boiled water surfaced nine times in the next half-hour and that in unboiled water only once. This seems to show conclusively that part of the oxygen requirements of *Xenopus* tadpoles are normally obtained from the water. In an exactly similar test on other tadpoles of the same size and age carried out 4 hr. later at a temperature of 17°C. the tadpole in boiled water surfaced four times in 43 min., but the one in unboiled water surfaced only once. The following morning the boiled water was aerated and the tadpoles which had differed at 6 p.m. each surfaced three times in half an hour, at 17°C. As a further test, two 8-day tadpoles each were placed in 300 ml. flasks of boiled and unboiled water and the flasks were then sealed without air space. Six and a half hours later the tadpoles in the boiled water looked moribund and were transferred to fresh water; they gulped hard for some time at the bottom before they could rise to the surface, but eventually recovered. The tadpoles in the unboiled water remained active for 48 hr. without the seal being broken, although they could not surface. The water by then was rather foul. This test seemed to show that the oxygen requirements of young *Xenopus* tadpoles are probably very low, since the small tadpoles remained healthy for a considerable time in boiled water without access to the air. Large tadpoles did not survive equally well without access to air, even though their water was partially aerated by a constantly dripping tap; two large 25 mm. tadpoles in 1 l. of water, prevented by a mosquito net from rising to the surface, died overnight. The temperature was about 23°C. It was thought that with more water under cooler conditions they would have survived longer, and a further experiment confirmed this. A single 27 mm. tadpole was put into 2 l. of water under a mosquito net and the jar was left under a dripping tap at a temperature of 16–17°C. This tadpole, though at first somewhat distressed, lasted 24 hr. and was taken out alive and survived—further proof that oxygen requirements can be satisfied under water. It is possible that the vibration of the tail fin contributes to the respiratory exchange.

*Feeding.* In our hands the feeding of algal or infusorial cultures was not very effective, and was obviously impractical on a large scale. We had not at that time seen Gasche's [1943] paper describing the use of dried clover and nettle, but following an American paper dealing with *Rana pipiens* tadpoles an experiment was carried out with liver powder made by grinding up fresh rabbit liver with a small amount of flour, drying in thin layers on sheets of glass in warm air, and grinding to pass a 60-mesh sieve. Such a dry powder keeps indefinitely in a stoppered bottle, and has the great advantage over Elkan's fresh liver emulsion of not fouling the water if dosage is adjusted to consumption.

An experiment with this powder (Exp. 1) was carried out as follows: fifty tadpoles 1 day after hatching were put into each of three tanks containing 12.5 l. of water. Tank A received 50 ml. of hay infusion daily; tank B received 50 mg. of liver powder daily, while tank C received no food. Two weeks later the tadpoles were counted and measured with the results shown in Table 1.

Table 1

Tank	No. of tadpoles remaining	Approximate length (mm.)				
		8-10	10-12	12-14	14-16	16-18
A	19	1	18	—	—	—
B	42	—	3	9	26	4
C	35	7	28	—	—	—

The superiority of the tadpoles in tank B was very obvious, and the mortality was low considering their small size when the experiment started. From the fact that the mortality in tank A was much greater than that in the unfed tank C it must be concluded that hay infusion as used by us was most unsatisfactory. Following this experiment, dried liver powder became our standard food for *Xenopus* tadpoles; dried ox liver prepared on a large scale and found to be equally good was substituted for rabbit liver when the demand increased. Our results with liver powder have since been confirmed by Landgrebe & Samson [footnote to 1944 paper], and in view of Gasche's results it is likely that a great variety of substances could be used successfully in fine powder form. Dried clover and nettles have a relatively high protein content, which may explain their efficacy.

In order to estimate the optimum dosage of liver powder, forty tadpoles which had been free-swimming for about 12 hr. were put into each of five 10 l. glass tanks A-E giving a density of four per litre (Exp. 3). These tanks received 10, 20, 40, 80 or 160 mg. of liver powder daily, the water being unchanged. After 10 days the tadpoles were measured. The results are tabulated in Table 2. The young tadpoles,

Table 2

Tank no.	Daily amount of liver powder mg. per tadpole	Average length of tadpoles after 10 days
		mm.
A	0.25	10
B	0.5	10
C	1.0	12
D	2.0	13.5
E	4.0	23

kept at a density of four per litre, thus showed most rapid growth on 4 mg. of liver powder per tadpole daily, though the water became somewhat cloudy in this tank.

Further experiments were carried out in small bowls. Ten tadpoles, 12 days old, were put into each of three 2.5 l. bowls and given 2, 4 or 8 mg. per tadpole of liver powder daily. All grew about equally well (average length at 22 days = 23.9–24.9 mm.), although the daily dose per litre varied from 8 to 32 mg.

In a similar experiment with ten tadpoles in each of three 1.25 l. bowls the average size at 22 days was 18.6 mm. on 2 mg. per tadpole daily and 23.8 mm. on 4 mg. per tadpole daily. Here there were eight tadpoles per litre and the daily concentration was 8 and 16 mg. per litre of liver powder. With higher concentrations of liver powder in the small bowls the water became foul. Fair results had previously been obtained with 1 mg. per tadpole per day (Exp. 1) and it was concluded that 2–3 mg. per tadpole per day was a satisfactory dose, allowing for good growth and not fouling the water. This conclusion was fully confirmed in later experiments, but it is likely that the assimilative powers of the tadpole vary both according to the vigour of the batch and to the temperature of the room. Moreover, this experiment did not show whether the important factor was absolute amount of liver powder available per tadpole or the concentration of powder in the water. It would be expected that a low concentration of food in the water, caused by feeding the standard amount of liver powder per tadpole to tadpoles at very low density, would result in an adverse food intake:energy-expenditure ratio and therefore in slow development. However, some of the liver powder gradually sinks to the bottom of the container and is grazed by the tadpoles, so that the importance of the concentration factor must depend partly on the shape and size of the container. Certainly no adverse effect of low concentration was seen in the first of the combined density and food supply experiments recorded below.

*Density.* Previous workers have shown that the number of tadpoles per litre is an important factor in growth, since there is a specific overcrowding effect, not connected with the toxicity of excretion products. The following experiment (Exp. 9) was carried out. Various numbers of small tadpoles, 9 days old, were distributed in 2 l. jars and fed at the rate of 1 mg. per tadpole per day. Results are given in Table 3.

Table 3

Jar no.	Total no. of tadpoles	No. of tadpoles per l.	Daily amount of liver powder mg. per l.	Average length of tadpoles after 13 days mm.
A	2	1	1	34.0
B	4	2	2	32.6
C	8	4	4	25.2
D	16	8	8	25.6
E	32	16	16	24.2

The growth in all jars was good, but the results indicate that under these conditions it is possible to get good growth at a low density per litre on a standard dose of as little as 1 mg. per tadpole daily. Low density of tadpoles was advantageous, and in this experiment was more important than concentration of food per litre. This result was confirmed in another experiment in which the concentration of food was kept the same but the density of tadpoles (6 days old) was progressively increased

(Exp. 8) Each of five 10 l. tanks had 160 mg. of liver powder daily, a concentration of 16 mg. per litre; the numbers of tadpoles, their density per litre and the amount of liver powder per tadpole varied as in Table 4.

Table 4

Tank no.	Total no. of tadpoles	No. of tadpoles per l.	Daily amount of liver powder mg. per tadpole	No. of tadpoles surviving	Average length of tadpoles on 13th day mm.
A	10	1	16	10	25.8
B	20	2	8	20	21.7
C	40	4	4	37	19.3
D	80	8	2	76	15.6
E	160	16	1	148	15.5

Although tank D was getting 2 mg. of liver powder per tadpole daily, growth was hardly better than in the tank E receiving half that amount. Growth in tank C, which corresponded to tank E in Exp. 3, was less rapid than in the previous experiment. The results for tanks A and B show that tadpoles at a low density can grow more rapidly and assimilate more food than tadpoles which are more crowded. Our results fully confirm those of Adolph [1931] that overcrowding results in great variation in size, and it is evident that for obtaining reasonably uniform and well-grown tadpoles suitable for assay work adequate 'lebensraum' is very desirable.

In a final experiment (Exp. 19) an attempt was made to overcome the effect of overcrowding by increasing the dose of liver powder per tadpole. Five jars (A-E) of 1½ l. capacity were set up containing respectively 5, 10, 20, 40 and 80 tadpoles which were fed so that the average daily amount for each tadpole in the different tanks was respectively 1, 2, 3, 4 and 5 mg. The tadpoles were 4 days from hatching at the start of the experiment. They were measured after 10 days, with the results shown in Table 5. Tank E rapidly became foul, and had to be discarded after 6 days.

Table 5

Jar no.	Total no. of tadpoles	No. of tadpoles per l.	Daily amount of liver powder		Average length after 9 days mm.
			mg. per tadpole	mg. per l.	
A	3	2	1	2	16.0
B	6	4	2	8	20.4
C	12	8	3	24	25.0
D	24	16	4	64	20.5
E	48	32	5	160	—

The surviving tadpoles in this tank showed very little growth. After 9 days the tadpoles in tank A were poorly grown, being different in this respect from the low concentration tadpoles in Exp. 9; the difference is probably accounted for by their difference in age. In tanks B and C, which both remained quite clear, the tadpoles were lusty, though those in C were better grown on the average. Tank D was turbid all through the experiment, and the tadpoles, although fairly long, were thin and unhealthy. A dose of 4 mg. per day per tadpole in other experiments with lower density of tadpole had been quite compatible with the water remaining clear. There is thus conclusive evidence of the reduced food consumption of crowded tadpoles.

No doubt the constant disturbance resulting to each individual from overcrowding means a constant interruption of feeding, which does not take place when the tadpoles are in rapid movement.

It should be noted that food supply and density experiments have related only to the early phases of growth which have been most important for our work. No doubt with larger tadpoles optimal density would be less and optimal food supply per tadpole greater. It may be assumed, however, that the same general principle holds at all stages—food supply should be maximal compatible with the water remaining clear.

*Shape of container.* Owing to shortage of aquarium tanks, containers of various shapes were used, and early in the work it was noticed that tadpoles in tall round vessels grew better than those in rectangular ones. A definite experiment was therefore carried out (Exp. 6). Forty tadpoles were put into a large filtrate jar, and forty others into a small rectangular tank; each contained 10 l. of water. The tadpoles had been free-swimming for 1 day at the beginning of the experiment. Each vessel received 80 mg. daily of liver powder, and all tadpoles were measured 14 days from the beginning of the experiment. In the jar the average length of the thirty-seven remaining tadpoles was 19.6 mm., and half of them were over 20 mm.; in the tank the average length of the forty tadpoles was only 16.6 mm., representing a considerable difference in the actual size of the animals. None was over 20 mm. long.

The experiment was repeated with the same vessels and the same volume of water, but with fifty tadpoles and 140 mg. of liver powder daily; after 10 days the average length of fifty tadpoles in the jar was 22.8 mm. and of forty-seven tadpoles in the tank 17 mm., a very striking difference. In the tank less than 11% of the tadpoles were over 21 mm.

Yung [1885], who kept his *Rana esculenta* tadpoles in cylinders of different height and diameter, attributed the differences in growth to variation in air surface. This explanation, though possibly correct for the *Rana* experiment, is not acceptable for the *Xenopus* experiment, since the surface area of the tank used in the above experiments (578 sq.cm.) was greater than for the cylinder (416 sq.cm.). The only explanation that can be offered for this result seems to be that tadpoles can move, swim, and feed with less disturbance in a round vessel.

In view of Yung's results a series of experiments were arranged (Exps. 11, 12, 15, 17) in which 2 l. of water were put into each of several circular vessels of heights and diameters shown in Table 6. Sixteen free-swimming tadpoles were put into each of these jars and were fed liberally on liver powder, as shown in Table 7. Length measurements were made after various periods.

Table 6. *Approximate measurements of the diameter of the vessels and the depth of the water (Exps. 11, 12, 15, 17)*

	Diameter (cm.)	Depth (cm.)
Cylinder E	8	40
Jar A	12	17
Jar B	15	12
Jar D	19	7
Petri dish C	22	5

Table 7

Exp. no.	Days on experiment	Daily food mg.	Average length (mm.) and numbers of tadpoles					Days old when measured
			E	A	B	D	C	
11	14	70	—	22.5 (15)	27.6 (16)	—	20.7 (16)	18
	24	70	—	32.1 (12)	35.1 (16)	—	26.4 (14)	28
12	15	70	26.2 (16)	—	22.0 (16)	20.4 (15)	18.6 (14)	22
	21	70	31.8 (16)	—	27.0 (16)	23.7 (14)	20.0 (14)	28
15	7	40	23.4 (16)	18.6 (16)	16.1 (16)	18.3 (16)	15.2 (16)	12
17	16	50	19.2 (16)	21.0 (15)	18.7 (16)	—	13.4 (13)	20

In Exp. 11 the best growth was in the vessel in which the height of the water approximated most closely to the diameter of the surface, but the tadpoles in the other jar were but little behind. Those in the Petri dish were noticeably backward. In Exp. 12 the average length of the tadpoles varied with the height of the vessels, and the tadpoles in the measuring cylinder E were larger than in any of the other vessels, those in the Petri dish again being smallest. The experiment was repeated (Exp. 15) with a reduced amount of liver powder on the assumption that the tadpoles in the shallow dish might have suffered unduly from the debris and faeces on the bottom. Once again, however, the tadpoles grew best in the tall cylinder and least well in the shallow water. In the fourth experiment the tall jar gave the best result and the Petri dish again the worst. There is at present no explanation of this curious phenomenon.

*Size of container.* Casual observation and the results of previous workers suggested that where density of tadpoles and food allowance were constant, tadpoles in small containers grew more rapidly than those in large containers. An experiment was therefore put up with different sizes of units, the tadpole density being constant at eight per litre and the food supply constant at 2 mg. per tadpole. Round receptacles of approximately the same shape were used. Results are shown in Table 8. No constant relation between size of tadpole and size of container is to be seen in these results. There was considerable variation in size of tadpole in each container and the smaller size of the four in jar A is of doubtful significance.

Table 8

Jar	Volume of water l.	No. of tadpoles	Liver powder per day mg.	Average size of tadpoles after 10 days mm.
A	$\frac{1}{2}$	4	8	16
B	1	8	16	19
C	2	16	32	20
D	4	32	64	18
E	8	64	128	21

*Disease.* *Xenopus* tadpoles, like the adults, seem to be remarkably free from disease. We have, however, had one or two attacks of 'tail-rot' caused probably by fungi of the genus *Saprolegnia*, members of which are responsible for a similar disease in fish. *Saprolegnia* species are usually said to be saprophytic so that they can obtain a hold only on dead tissue; in the case of fish wounded or damaged specimens are the usual victims. It is not certain that all the *Xenopus* tadpoles affected presented such a nidus, so that the tentative diagnosis may be mistaken. However,

a similar condition in *Rana* has been controlled successfully by the use of mercurochrome, to which our attention was drawn by Dr Hindle, and we expect to be able to deal with any further outbreaks in *Xenopus*. In the meantime, routine disinfection of containers is carried out each time they are emptied (10 drops of 1/200 solution of mercurochrome per gallon of water), a precaution which has been associated with absence of further outbreaks. It is possible that the adult toads used for breeding act as carriers.

### *Metamorphosis*

When the tadpoles reach a length of about 45–50 mm. the hind limbs, which undergo gradual and continuous development from an early state, are fully differentiated and well grown. The front legs, by contrast, develop under the skin, erupt suddenly and differentiate rapidly, but they are not well developed when the most striking phase of metamorphosis takes place—the shrinkage of the large transparent head. This process, which reduces the thorax to a fraction of its former size and involves retraction of the eyes and other complicated phenomena, occupies only 2 or 3 days. When the shrinkage of the head is completed, and not before, the tail is resorbed over a period of 2–3 days, and the animal assumes its adult form. The tadpole feeding mechanism disappears with the shrinkage of the head, and a day or two after metamorphosis is complete the new feeding habit appears—the toad will catch and swallow any small moving piece of meat, such as a young tadpole. Very soon it readily takes bits of liver and thereafter can be fed exclusively on this diet. Under the conditions existing in our colony the completion of metamorphosis has occurred as early as 6 weeks after oviposition, though there is great variation among tadpoles even of the same batch kept in the same tank. For instance, some fifty tadpoles were kept back from a large batch used for assay work and allowed to grow. The first had completed metamorphosis by the 42nd day, within another 2 weeks ten more had metamorphosed, and within 3 months of oviposition all had completed metamorphosis except a few stragglers. Metamorphosis occurs earlier where the population is less dense, and the large tadpoles which result from their having adequate space give rise to large toads which thrive well.

### *Rearing of young toads*

When the tail begins to shrink the young toad is removed from the tadpole tank to a small jar containing a few inches of water, so that it can receive individual attention over the critical stage of the change of feeding habit. Within a week or two, when the animal is well established and thoroughly accustomed to eating liver, young toads of the same batch are collected together in tanks, about ten per square foot of tank area, and reared as described by previous workers. In the early stages it is desirable to feed them every day, but the interval can be increased as the toads get bigger. Unless a running water system is provided (as described by Landgrebe & Samson), the water, which is fouled by the liver and faeces, should be changed shortly after feeding. We have not tried any form of meat other than liver, in view of the results of Landgrebe [1939] who found that liver was superior to beef, especially if the intervals between feeding were long. This author concluded that the efficacy of liver was not due to its high manganese content, and was not increased by the addition of various vitamins. On liver supplemented by tadpoles, five female toads



from one of our batches attained an average weight of 17 g. in 8 months from oviposition. Landgrebe & Samson record that young toads grew much more rapidly when fed on live *Lebistes* than when fed on liver. We have no precise information about this superiority of live meat, but it was noticeable that our young toads showed very good growth during the time when the liver was being supplemented by some thousands of *Rana* tadpoles. On the other hand, the growth of our young toads on liver alone seems to have been much better than that recorded by Landgrebe & Samson, probably because of daily feeding and higher temperature.

The sex of the adults is easily diagnosed from the prominent cloaca of the female. In large toads, body size is also diagnostic since males rarely exceed 40 g. Females are more generally useful than males, and it would be desirable, in a breeding colony, to be able to distinguish and discard males at an early stage. The cloaca often becomes evident in very small females, but in other individuals there is greater difficulty in distinguishing the sex. By the time the toads weigh 10 g., however, diagnosis in doubtful cases can be made by the injection of 200 i.u. of chorionic gonadotrophin, which causes the cloaca to enlarge in the females. Landgrebe & Samson report that oviposition can be induced in females as small as 16 g. weight, but we have not so far been successful with toads of this size. By contrast, a young male of 20 g. has proved to be an excellent stock-getter. We have not determined with any precision at what stage the sex dimorphism in body size begins to appear; on the whole our observations suggest that both sexes grow at about the same rate during the immature period. Thus, of twenty toads raised from the same batch of eggs and weighed 8 months after oviposition, fourteen females and the six males had the same average body weight of 13 g.

#### RESPONSE OF *XENOPUS* TADPOLES TO THYROIDAL ACTIVITY, AND TECHNIQUE OF ASSAY

##### *Introduction*

The assay of thyroidal activity by the induction of premature metamorphic changes in tadpoles has been discussed in a previous paper [Deanesly, Emmett & Parkes, 1945]. Most of the earlier work has been done on tadpoles of species of *Rana*, but the majority of these suffer from the disadvantage of being available only at one time of year, and this still applies to *R. temporaria* tadpoles in Great Britain. Since *Xenopus* can be made to produce fertilizable eggs at any time, an investigation was made of the suitability of the tadpoles for the assay of thyroidal activity. It was found that their response was well defined and could be more readily assessed quantitatively than that of *Rana*, so that, quite apart from the question of availability, *Xenopus* tadpoles had great advantages for the test.

The changes occurring in *Xenopus* tadpoles after the administration of preparations with thyroidal activity, the selection of the particular change most suitable for quantitative work, the variables associated with its use as a test object for assay, and the application of the test are described below.

*Handling of tadpoles*

All the details of feeding, rearing, etc., can be found in the preceding sections of this paper. For assay purposes, the tadpoles are mainly used between 20 and 25 mm. in length, which the majority attain 2-4 weeks after hatching under good conditions of food supply and temperature. All batches contain a small proportion of outstandingly vigorous growers; these are not used for assay but are put aside for rearing. Retarded stragglers, also found in most batches, are discarded. The tadpoles are not fed while under test. Other details of the maintenance of the animals on test are given in the appropriate place. Tap water treated with powdered liver, but without obvious suspended matter, as described above, was used for all tests. When necessary for grading and record purposes measurements of total length were made on the anaesthetized tadpoles in a small Petri dish over squared paper.

*Substances used*

The following substances were used in the work dealing with the variables of the *Xenopus* test.

*Dried thyroid.* Two preparations, made by Boots Pure Drug Co., both containing 0.09-0.11 % thyroxine iodine corresponding to a thyroxine content of about 0.15 %.

*Thyroxine.* Unless otherwise stated the thyroxine used was synthetic *DL*-thyroxine made by British Drug Houses. Some experiments were carried out with *l*(-)-thyroxine.

*Iodinated casein.* Three preparations were used, NC3, NC4+5 and PB11. The preparation of NC3 is described by Pitt Rivers & Randall [1945]. It contained 2.4 % acid-insoluble ('thyroxine') iodine. NC4+5 was compounded of 22½ lb. of NC4 and 30 lb. of NC5, both of which preparations are described individually in the paper by Pitt Rivers & Randall. NC4+5 contained 1.6 % acid-insoluble iodine. PB11, made by I.C.I. (Explosives) Ltd., contained 3.3 % acid-insoluble iodine.

*Di-iodotyrosine, di-bromotyrosine, and di-chlorotyrosine* were prepared from natural *l*(-)-tyrosine.

*Seaweed meal* was obtained from Mr A. P. Orr.

*Changes produced in Xenopus tadpoles by administration of substances with thyroidal activity and criteria of response for assay purposes*

Preliminary experiments showed that marked changes could be produced in medium-sized *Xenopus* tadpoles by exposure to small amounts of thyroxine, dried thyroid, or artificially iodinated proteins. These changes are essentially similar to those seen at normal metamorphosis, but the order of appearance is somewhat different. In normal metamorphosis the order is: growth of hind limbs, eruption of front legs, shrinkage of large transparent head, and, finally, absorption of the tail. When a thyroid-active substance is given to young tadpoles the head shrinks very quickly, and the front legs erupt while the hind legs are still very small. There is no immediate absorption of the tail, but the other changes appear in a few days under suitable conditions. Their relative values as criteria of response for assay purposes were investigated on tadpoles about 20-30 mm. in length.

*Changes in total length.* Separate measurement of the body and tail was not found to be practicable, and attempts were made to measure total length. This is difficult to do with accuracy, even under anaesthesia, owing to the tail being semi-transparent

and curved. In a first experiment tadpoles lost about one-quarter of their length following treatment up to 15 days. In a second, slight reduction in length began 3 days after the start of heavy dosage with iodinated casein, but after a week the average decrease was only about 15%. This experiment was carried out at room temperature and a greater response might have occurred at a higher temperature. However, the heavy dosage was proving lethal after 7 days, and would have been fatal even earlier at a higher temperature. Attempts to use changes in total body length were then abandoned.

*Changes in the head.* The head of the normal *Xenopus* tadpole is almost rectangular seen from above (Pl. 1, fig. 1) and is wedge-shaped in profile. The shrinkage following exposure to thyroidal substances makes the head triangular in shape from above, the apex being towards the tail, and the front of the head becomes 'dished' in profile, acquiring a curious bulldog appearance. The eyes, though actually drawing closer together, protrude from the surface of the head. These changes are very obvious to look at but very difficult to assess quantitatively. Attempts were made to measure the anterior-posterior length of the head and the interocular distance by means of a micrometer binocular microscope. This technique required a degree of anaesthesia (under chlorotone) which was often fatal, and the results were, in general, unsatisfactory.

*Changes in the hind limbs.* Hind-limb buds are present from an early age, and measurement of their growth was attempted under the binocular microscope, but again anaesthesia was required and experience showed that the method was impracticable for large numbers.

A curious phenomenon was noticed in connexion with induced growth of the hind limbs. In many instances, vigorous stimulation by a heavy dose resulted in the appearance, just before the differentiation of the digits, of a highly vascular area near the distal end of the leg (Pl. 1, fig. 2). It showed as a bright red spot, varying up to about 1 mm. in diameter, visible with the naked eye, though more clearly seen under a hand lens. This spot lasted only 1-3 days, sometimes on one, sometimes on both, legs. The use of a transient reaction for biological assay is quite practicable (vaginal cornification in mice for instance), though it has the disadvantage that the animals must be individually identified. A systematic attempt was therefore made to see whether the appearance of the vascular spot was sufficiently regular, and correlated with the dose, to be used as a quantitative criterion of response to thyroidal substances.

In the first experiment (test 6) thirty tadpoles, 20-25 mm. in length, and each in a separate container holding 220 ml. of water, were divided into six groups of five. One group received no treatment and showed no reaction. The remaining five groups were dosed by adding to the water varying amounts of iodinated casein (NC3) which previous tests on *Xenopus* had shown to be about as active as the usual preparation of dried thyroid. The water and the dose were renewed each day. The earliest spots appeared on the second day after dosing. No new ones appeared after 4 days. A well-defined macroscopically visible spot was required for a positive, and there was little difficulty in assessing borderline cases. In the most heavily dosed groups three tadpoles died before they could have responded, and are omitted from Table 9 which shows the results of the experiment.

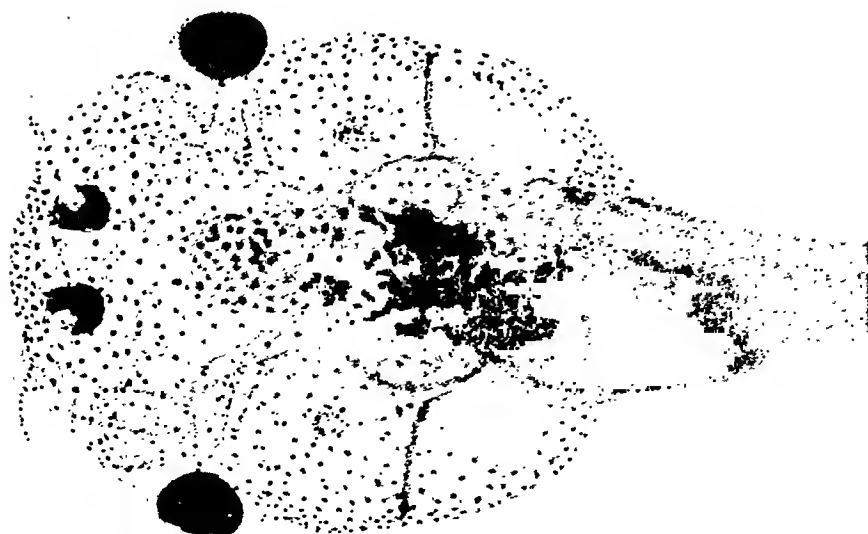


FIG. 1. Head and body of normal *Xenopus* tadpole,  
22 mm. in length.



FIG. 2. Head and body of similar tadpole  
after treatment with iodinated protein, show-  
ing vascular spot at end of right hind limb.

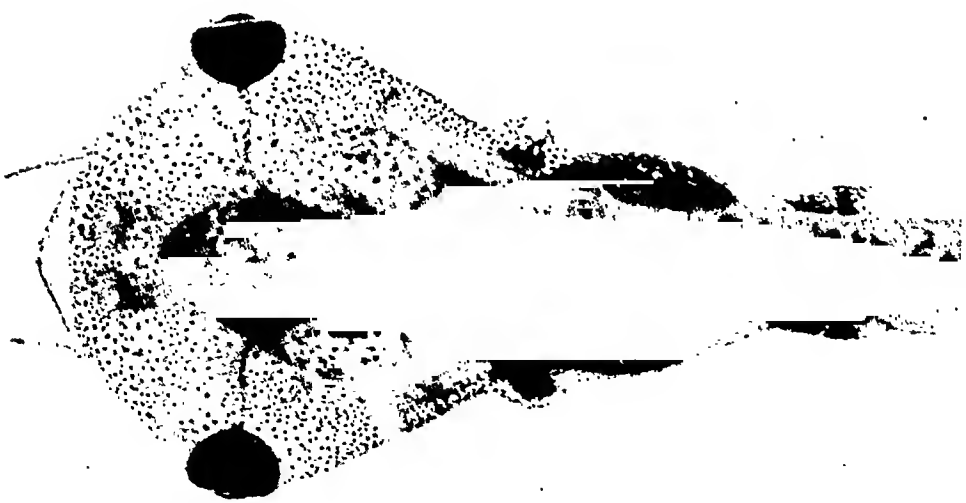


FIG. 3. Front-limb buds showing under the skin.

Head and body of tadpoles after treatment with iodinated protein, showing wastage of head, development of front limbs, and growth of hind limbs.



FIG. 4. Right front limb on verge of eruption.



FIG. 5. Left front limb erupted. Positive result.

FIG. 6. Both front limbs erupted.  
Head and body of tadpoles after treatment with iodinated protein, showing wastage of head,  
development of front limbs, and growth of hind limbs.



Table 9. *Appearance of vascular spot on hind legs*

Dose (mg.) of NC3	No. of tadpoles	Days after dosing						Total response
		2		3		4		
		L	R	L	R	L	R	
8	32/2	—	—	+	+	—	—	3/3
	32/3	+	+	Died		—	—	
	32/4	—	—	—	—	+	+	
4	33/1	—	—	+	+	—	+	4/4
	33/2	—	—	+	+	—	—	
	33/4	—	—	—	—	+	+	
	33/5	—	—	—	+	Died		
2	34/1	—	—	+	—	+	—	5/5
	34/2	—	—	—	—	—	+	
	34/3	—	—	—	—	+	—	
	34/4	—	—	+	+	+	+	
	34/5	—	—	—	—	—	+	
1	35/1	—	—	—	—	—	—	2/5
	35/2	—	—	—	+	—	+	
	35/3	+	+	—	+	—	+	
	35/4	—	—	—	—	—	—	
	35/5	—	—	—	—	—	—	
0.5	Five tadpoles, all negative							0/5

L=left leg; R=right leg; +=vascular spot; —=no vascular spot.

There was a clear relation between dose and response in this first experiment, and the method appeared to be promising in spite of the fact that few of the animals getting an effective dosage survived beyond the fifth day. Later experiments carried out with thyroxine and dried thyroid in addition to iodinated casein NC3 were not very successful and showed a less satisfactory relation between dose and response. In view of the high dosage and the transience of the reaction, necessitating individual identification and repeated examination of the tadpoles, experiments on these lines were abandoned.

*Eruption of the front legs.* Observations were next made on the eruption of the front legs. One of the early signs of thyroidal stimulation of young *Xenopus* tadpoles is the development of the front-limb buds, which appear in the branchial sac under the transparent skin on each side of the body anterior to the gut (Pl. 2, fig. 3). The bulges thus formed enlarge (Pl. 2, fig. 4) until the legs, usually undifferentiated, erupt and project at right angles to the body as small white stumps which can readily be seen with the naked eye (Pl. 3, fig. 6) and can be examined closely under a hand lens. In this species [Bles, 1905] the forelimb develops in a diverticulum of the branchial chamber which becomes shut off from it. When the front limb emerges the spiracles remain unaffected and the animal continues to breathe and feed by means of the current of water. From the point of view of assay, this occurrence in the stimulated tadpole has a number of interesting characteristics. In the first place, it happens fairly suddenly, and although, before they break through, the limbs can be seen protruding under the transparent body wall, there is very seldom any doubt as to whether or not one has actually erupted. The reaction, therefore, forms an excellent qualitative criterion of response, and the response of the group as a whole on a given day can be taken as the percentage of tadpoles responding. Often one



leg erupts before the other (Pl. 3, fig. 5). Such a unilateral response could, for statistical purposes, be considered as positive or negative; we have taken it as positive. A tadpole dying before the end of the test is excluded from the calculations. Secondly, since the reaction is irreversible, individual identification is not necessary, and the test tadpoles can be kept together in any convenient size of unit. Thirdly, the premature eruption of the front legs in *Xenopus* tadpoles does not cause death. This fact removes what in the case of *Rana* [Deanesly, Emmett & Parkes, 1945] is a serious objection to the use of front leg eruption for assay purposes. Fourthly, after a limited period of dosage, the legs erupt, if at all, without much delay.

*Variables in the test based on the eruption of the front legs*

Early experiments gave promising results on the use of the eruption of the front legs for quantitative work, but showed that the reaction was much affected by several variables, some of which have been investigated in detail. In the account which follows, the technique, except where otherwise stated, has been to put five tadpoles in 200 ml. of water containing the required dose of iodinated protein in suspension, change to fresh water after 3 days and observe the result on the seventh day.

*Method of administration.* The routine method of administration was as follows. The iodinated casein was ground until it would pass a 90-mesh sieve; the weighed amounts of the powder were ground with water and made up as a suspension containing a suitable amount per ml. The suspension was added to a beaker containing the tadpoles, and the total volume was made up to 200 ml. A similar method was used for thyroid powder, and for thyroxine with the addition of a trace of alkali. This technique was highly effective and gave a clear-cut relation between dose and response, but since little, if any, of the iodinated protein passed into true solution under the conditions stated, it raised the question of whether the active material was absorbed or ingested by the tadpoles. This question was the more important because Reineke & Turner [1942] found that *Rana pipiens* tadpoles gave little or no response to iodinated casein added to the water and they adopted a micro-injection technique. Thyroxine, moreover, is not active in mammals when ingested by mouth. Injection of *Xenopus* tadpoles seemed to be impracticable, but experiments were carried out to see whether the iodinated casein would be more effective if added to the water in true solution, or if not, whether particle size in the suspensions was an important factor. In the first experiment, suitable doses of iodinated casein PB11 and iodinated casein NC3 were added to the water (*a*) by the usual technique of suspension, and (*b*) after being put into solution by adjustment of the pH to 9.0 by the addition of borate buffer and incubation at 37°C. Other conditions were constant. At the time this experiment was carried out the pH of the livered tap water was about 8.4 and the addition of 4 ml. of the buffered solution to 200 ml. of water raised the pH to 8.8. Very little of the iodinated protein was precipitated by mixing the solution with the livered tap water. The results of this experiment (Table 10) were consistent in showing the material to be more effective when added to the water in the form of solid particles. PB11 was less than one-fifth as active when used in solution. The experiment may have been complicated by some destruction of the protein during incubation at pH 9.0, by the addition of borate to the medium,

Table 10. *Relative effect of suspensions and solutions*

Substance	Condition in which added to beakers	Dose mg.	No. of tadpoles	% positive response	Relative effectiveness*
Iodinated casein PB11	Aqueous suspension	1.0	10	70	1.0
		2.0	10	90	
	Solution at pH 9.0	1.0	10	0	0.2
		2.0	10	30	
Iodinated casein NC3	Aqueous suspension	0.5	20	30	—
	Solution at pH 9.0	0.5	20	0	—

\* See section on statistical methods (p. 351).

or by the raising of the pH of the medium, but it is possible that the active material could not be equally well ingested in solution. In any case there seemed to be no advantage in trying to make solutions for routine testing of iodinated protein.

In a second experiment, 0.5 mg. of iodinated casein NC3 in aqueous suspension was added to livered tap water adjusted to various pH's between 7.58 and 9.04. The tadpoles, five in each group, all survived these comparatively alkaline reactions, but the number of positive responses was not significantly different in the different media and the variation showed no correlation with the pH. It is unlikely, therefore, that the exact pH of the medium is a substantial factor in the intensity of response. This conclusion is of some importance in view of the probable variation in the tap water and the lack of exact standardization in the amount of liver added to the water.

In a final experiment, various doses of iodinated casein NC4 + 5 were added to the water after being subjected to peptic digestion, treatment designed to break down the active material into a form soluble in water. The results, compared with a simultaneous assay of NC4 + 5 administered in aqueous suspension, are shown in Table 11.

 Table 11. *Effect of peptic digestion on the activity of iodinated casein*

Substance	Dose mg.	No. of tadpoles	% positive response	Relative effectiveness
NC4 + 5 in aqueous suspension	0.35	10	30	1.0
	0.50	10	50	
	0.70	10	100	
NC4 + 5 after peptic digestion	0.50	10	50	0.94
	0.70	10	90	

The apparent 6% difference in effectiveness is, of course, not significant, and it must be concluded that the active material made soluble by digestion is equally available to the animal or else that various factors cancelled out. This result is in contrast to that on the effect of making an alkaline solution and suggests that the low effectiveness of the latter was due to some destruction of the active material. However, the results of the digestion experiment show that, while there may be no disadvantage in making a solution for dosage, there is no advantage except perhaps a slightly increased accuracy of dosage. This rather surprising effectiveness of active material in suspension is probably due to the fact that dried thyroid and the iodinated proteins are active by ingestion and that *Xenopus* tadpoles feed by filtering out particles of solid matter from the water. The activity of thyroxine administered in

the same way is more curious, though we have not precise information as to the relative effectiveness of this substance in true solution.

*Concentration and absolute amount in dosage.* The feeding experiments described previously showed that the growth of the tadpole was affected under certain circumstances by the concentration of food particles in the water as well as by the absolute amount available per tadpole. Since the iodinated casein seemed to be taken in largely by ingestion it was desirable to investigate the relative importance of these two factors in influencing the effectiveness of a given dose of iodinated protein. In an early experiment, it was found that five tadpoles in 200 ml. of water dosed with 1.0 mg. of iodinated casein NC3 responded as well as five tadpoles in separate similar beakers. This dose was rather high, giving 100 % response at 7 days, but the result made it most unlikely that the effectiveness of a dose of this size put into the water would decrease proportionally to the number of tadpoles in the beaker.

An experiment was carried out later which showed clearly that concentration of the dose was an important factor in response. Receiving the standard concentration were (A) one 2 l. Petri dish with ten tadpoles; (B) two 400 ml. beakers each with five tadpoles; and (C) two 200 ml. beakers each with five tadpoles. Receiving a standard amount per tadpole were (C) two small beakers as above; (D) two 400 ml. beakers each with five tadpoles; and (E) a large 2 l. Petri dish with ten tadpoles. The percentage response is shown in Table 12. A dose (0.1 mg. per tadpole) which

Table 12. *Relative effect of concentration and absolute amount of dose*

	Amount per l. mg.	Amount per tadpole mg.	Volume per tadpole c.c.	% response
A	2.5	0.5	200	55
B	2.5	0.2	80	30
C	2.5	0.1	40	44
D	1.25	0.1	80	0
E	0.5	0.1	200	0

produces a medium response when the concentration is 2.5 mg. per litre fails to do so when the volume of the medium is doubled. Conversely, increasing the volume of the medium at the concentration of 2.5 mg. per litre does not increase the response. This effect of concentration is undoubtedly due to the fact that the amount of active material a tadpole can absorb or filter out of the water will depend primarily on the concentration in the water and also, of course, on the activity of the tadpole. The absolute amount present in the container will exert an effect only in so far as removal of material by the tadpoles will lower concentration less rapidly when at a given concentration there is a larger amount of medium per tadpole. The residue experiments described below show that, under the conditions used by us, the concentration required to enable the tadpoles to obtain enough active material for a positive response is not quickly lowered by the amount removed by the tadpoles. Probably such a decrease would only occur rapidly with a very small volume of medium, at standard concentration, per tadpole. Thus, under the conditions of the test, concentration may be regarded as the important factor and dosage has, therefore, been expressed for assay purposes as the amount of substance added to the standard container holding five tadpoles in 200 ml. and not as the amount per tadpole.

With the best preparations of iodinated proteins used, the effective concentration was of the order of 1/400,000.

Another aspect of the dosage problem was studied by estimating the residual activity in the suspension after the tadpole or tadpoles had been in it for 3 days. In the first experiment, 2 mg. of NC3 were put into each of five beakers with 220 ml. of water and one tadpole. After 3 days the tadpoles were removed to fresh water and new tadpoles substituted in the once-used medium. This procedure was repeated eighteen times, fresh water being added to make up for evaporation. Positive responses, fifty-six in all, were obtained among each of the first sixteen sets of tadpoles. In other words, the active substance was still at an effective concentration even after fifteen successive tadpoles had spent 3 days each in it. Incidentally, it is of interest that the iodinated casein retained its activity after 50 days in water at 70-78°F. when mixed up with tadpole faeces, and that 220 ml. of water did not become too foul for the tadpoles after several weeks.

Several further experiments on residual activity were carried out with smaller doses and with five tadpoles per beaker. In the first two, with 0.5 and 0.7 mg. doses, the second set of tadpoles responded as well as the first; the third set did not respond. These doses, therefore, had been less than half used by the first set of tadpoles. The experiments again emphasized the importance of concentration, since one-half of the smallest dose used (0.25 mg.) is not itself an effective dose. At a higher temperature there seems to be greater consumption of the dose of active substance, since in later experiments at 27°C. there was no response in the second set of tadpoles subjected to the residue of an original dose of 0.4 or 0.6 mg.

*Duration of dosage.* It was necessary to decide on the optimal period of dosage to stimulate eruption of the front legs and whether during this period, if more than 1 day, the dose would have to be renewed because of its being unstable under the conditions existing or of its being used up by the tadpoles. Early experiments did not show any advantage in renewing the dose daily, and this conclusion was, of course, substantiated by the residue experiments described above. Since only one change of medium from dosed water to fresh water was thus required, it became immaterial as regards convenience whether dosage was for 1 day or longer, but it seemed likely that longer treatment would give positive results with lower dosage and make possible assays on small amounts of material. With a large dose a 1-day treatment may be effective. Doses of 0.5 and 2.0 mg., however, produced a better response when given over 3 days than over 2 days. Later experiments showed both

Table 13. *Effect of duration of treatment*

Substance	Dose mg.	Duration of treatment days	Day of observation	% positive response	No. of tadpoles
NC3	0.5	2	6	0	8
	0.5	3	6	0	9
	2.0	2	6	11	9
	2.0	3	6	50	6
NC3	0.5	3	7	30	10
	0.5	7	7	100	5
Thyroxine	0.05	3	7	80	10
	0.05	7	7	100	5

for iodinated casein and thyroxine that the response appeared more intensely if the tadpoles were left in the dose during the whole period of observation (Table 13), but it was considered that prolonged dosage would increase the number of deaths. Moreover, a restricted period of dosage would limit the time during which positive responses would occur, which was desirable, since readings are best made on the flat top of the time-response curve. A 3-day period of treatment was therefore adopted as a routine.

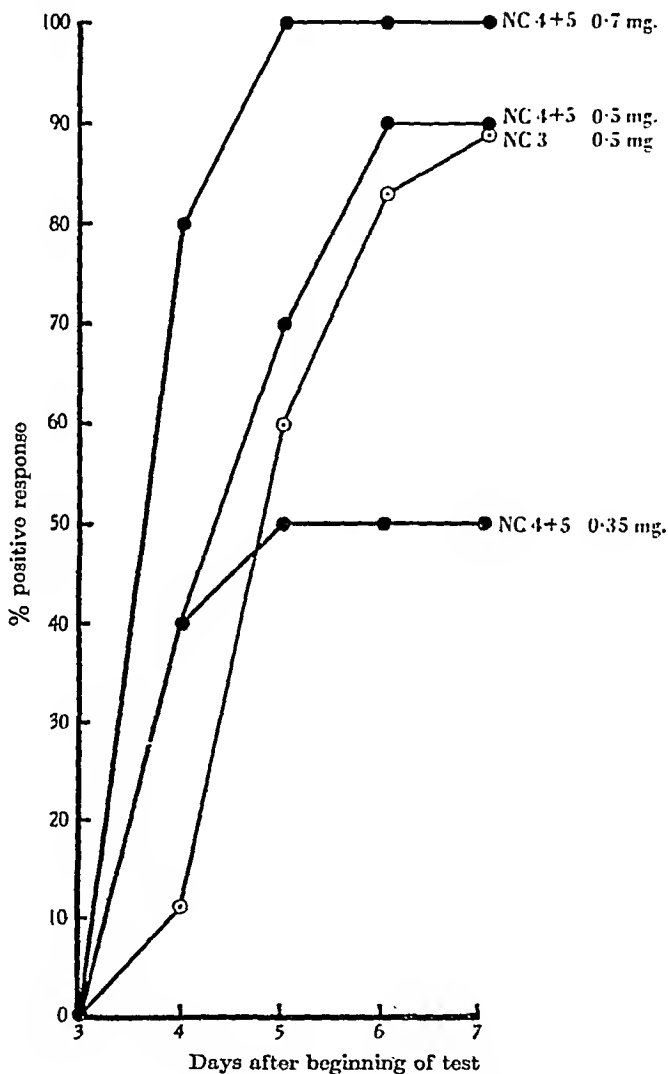


FIG. 1. Time-response curves for various doses of iodinated casein NC4+5 and one dose of NC3.

*Time of assessing response.* In deciding on which day to observe the response it was desirable to allow enough time for the full effect to appear, but otherwise to shorten the period of experiment as far as possible.

In many experiments, especially the early ones, observations were made on several consecutive days after treatment. These showed that except with high doses it was most unusual for any legs to have erupted by the time treatment ended, i.e. at the

end of the third day, and the response appeared mainly on the fourth and fifth days. Little additional response occurred on the seventh day, and in the cases where observations were continued additional response on the eighth and ninth days was lacking or negligible. As a routine, therefore, the final examination has been made, at the end of the seventh day. Some typical time-response curves are shown in Figs. 1 and 2, from which it will be seen that the response may be more rapid with higher doses, but even with small or medium doses it has usually reached its maximum by the end of the seventh day. This conclusion applies to the response to thyroxine and dried thyroid as well as to that to iodinated protein.

Other observations, not recorded here in detail, showed that the response was more rapid if treatment was continued after 3 days, but it is difficult to dissociate this effect from that which would be expected from the higher effectiveness of continuous treatment. The rate of response does not seem to be influenced by the size of the tadpole; certainly there is no appreciable decrease in the latent period over the range of size of tadpoles used by us for assay purposes.

In general, it may be said that a response which, for whatever reason, is low, appears later than one which is good, but that observation at the end of the seventh day records all but a very few positive responses. Certainly, by the end of the seventh day no rapid increase in response is taking place such as would magnify inaccuracies due to timing.

*Size and age of the tadpole.* All the tadpoles used were at least 1 month before natural metamorphosis and variations in response due to age or size within the chosen limits were not expected to be important. Nevertheless, tadpoles were carefully graded for test, similar groups being arranged for each separate dose. The usual procedure, as soon as a sufficient number of tadpoles in a batch were large enough to be used, was to select into a shallow dish those of convenient dimensions (usually 20-24 mm. but sometimes as small as 18 mm. or as large as 26 mm.) and grade them according to size. If twelve different doses were to be tested, then the twelve largest tadpoles were distributed into each of twelve beakers and so on for the next largest animals. By this arrangement each dose was tested on a group of tadpoles of about the same dimensions. Since each group of tadpoles commonly occupied two beakers

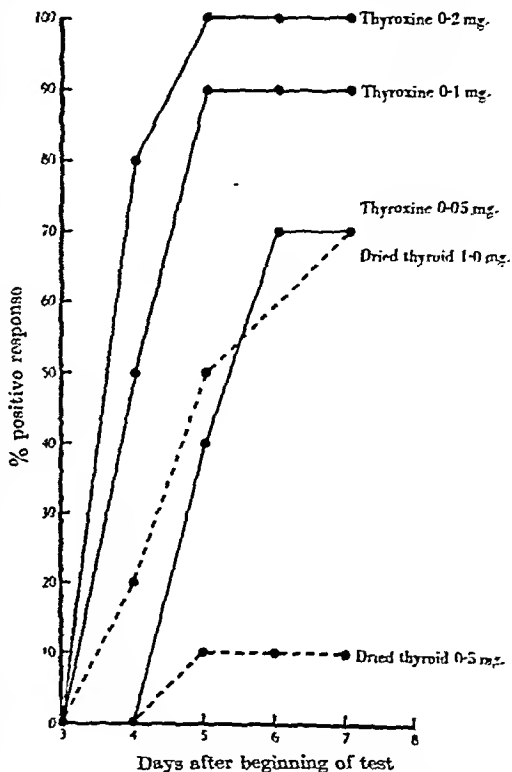


FIG. 2. Time-response curves for different doses of dried thyroid and thyroxine.

the first of these contained the largest animals on test; the response in these beakers could then be compared with the response in the second beakers of the pairs. No evidence was obtained by this method that the larger tadpoles were appreciably more sensitive than the smaller ones, or vice versa. In experiments with greater extremes of size and age, however, some suggestion of variation in sensitivity was obtained. It is clear that in any one test animals of the same batch can only be compared for size; if animals of the same size but of different age are to be directly compared they must be from different batches, which introduces a further variable. The results of two such tests (Table 14) indicate that on the whole the faster growing

Table 14. *Effect of age and size on response of tadpoles*

Batch no.	Size of tadpoles mm.	Age of tadpoles days	% response to NC4+5	
			0.4 mg.	0.6 mg.
23	27-21	41	30	60
23	21-20	41	0	20
23	20-19	41	0	10
25	24-21	14	60	100
25	20-21	15	100	100

animals show the highest response to thyroid-active preparations; the laggards in batch 23 which were still below 22 mm. at nearly 6 weeks old gave a low response as compared with the large ones in the same batch, and those in their turn gave a lower response than some fast-growing animals from a much younger batch. Possible differences in sensitivity between the tadpoles have been accentuated in these tests. Graded according to the usual methods, the smaller animals in batch 23 would have been discarded and the more actively growing tadpoles of batch 23 would have been put aside as growers or would have formed a small part of a larger test a week or so later. In general, therefore, it may be said that within the limits used, and under the conditions of our technique, age and size of tadpole is not an important variable, and with the system of grading described above is adequately controlled by the use of a standard preparation in each test. Variation from batch to batch may be more important, but it can also be controlled by the use of a standard preparation.

*Temperature.* Experiments showed that the response of the tadpoles depended to a large extent on the temperature of the medium during the test; below 20°C. the animals were comparatively unaffected by suspensions which would cause 100% response at 24-25°C. At the latter temperature the range of dosage for the standard iodinated casein preparation was 0.25-1.0 mg. per 200 ml. beaker, but in one experiment at room temperature, varying between 15 and 20°C., doses of 2, 4 and 8 mg. per beaker of the same preparation produced no responses. In another experiment tadpoles exposed for 24 hr. at room temperature to doses of the iodinated casein standard as high as 48 and 96 mg. gave no response. In general it is likely that *Xenopus* tadpoles are almost immune from the effects of thyroidal activity below 20°C., above which sensitivity rapidly increases so that at 24°C. the tadpoles respond to a fraction of the dose required at even 4°C. lower [cf. Reincke & Turner, 1942]. Within limits the intensity of the reaction can be regarded as a product of the dose and the temperature.

It soon became evident that a thermostated incubator would be necessary, and a test showed that the response was not affected by keeping the tadpoles in the dark. Ventilation of the beakers was initially thought to be desirable, and an egg incubator was suitably modified and adjusted to work at an air temperature of 27–28°C., giving a water temperature in the beakers of about 24.5°C. The temperature tended to rise temporarily when the drawer was opened to remove the beakers for changing the water or for examination, but this had no ill-effects on the tadpoles which would tolerate air temperatures up to 30°C. On the other hand, the varying duration of the temperature fluctuations, though not serious owing to the regular use of a standard preparation at each test, occasionally caused the results to be largely positive or negative, and therefore of little value for assay. Subsequently, when it became evident that ventilation of the beakers during test was not necessary, a large thermostatically controlled water tank was set up in which the beakers, carried on a wire-mesh tray, could be immersed up to a suitable level. The whole tank was covered with a metal lid. Under these conditions fluctuation in the beaker temperature during the 7 days of the test was less than 0.5°C. and more satisfactory results were obtained.

It was not thought necessary to undertake a complete investigation of the relation between temperature and response. In one test, two doses of NC3 were tested at 22 and at 25°C. At the lower temperature the substance was only about one-half as effective as at the higher one. On another occasion little difference was detected in the responses given at 24.5 and 25.5°C. Increase from 24 to 27°C. also caused little change in response. In six consecutive tests at 27°C. NC4+5 had an average M.E.D. of 0.57 mg., while in two tests at 24°C. the average M.E.D. was 0.46 mg. In a specific comparison on tadpoles of the same size and same batch the M.E.D. of NC4+5 was 0.42 mg. at 27°C. and 0.45 mg. at 24°C. It may be concluded, therefore, that sensitivity increases sharply with increase of temperature up to 24°C. above which, within limits, response is constant. It was noticeable, however, that there was much more wastage of the body at 27°C. than at 24°C.

### *Specificity of the Xenopus test*

The results recorded above show that *Xenopus* tadpoles respond vigorously and identically to *dl*-thyroxine and dried thyroid and to iodinated proteins of the type from which thyroxine has been isolated or which may be assumed to contain thyroxine. Experiments with other substances are discussed below.

*l*(-)-Thyroxine. Our experiments have mainly been carried out with synthetic *dl*-thyroxine. In tests on both rats and tadpoles, Gaddum [1930] found that *l*(-)-thyroxine was more active than *d*(+)-thyroxine, but that the latter showed definite activity. The same preparations were apparently equally active on myxoedema patients [Salter, Lerman & Means, 1935]. Later results have, however, shown that these preparations, both of which were obtained by resolution of synthetic thyroxine, were not optically pure. Foster, Palmer & Leland [1936] have since reported that *l*(-)-thyroxine obtained from the thyroid gland is twice as active on the B.M.R. of guinea-pigs as synthetic *dl*-thyroxine, a result implying that *d*(+)-thyroxine is inactive on mammals. We have had the opportunity of testing on *Xenopus* tadpoles a specimen of synthetic *l*(-)-thyroxine having the highest specific rotation yet re-



corded and have failed to find it more active than the *dl*-form; in three successive tests on more than 100 tadpoles the results were all compatible with the *l*(-)- and *dl*-forms being equally active, and incompatible with the *l*(-)-form having twice the activity of the *dl*-form. It is conceivable that tadpoles can utilize the *d*-form and that mammals cannot, but there does not appear to be any analogy for such an assumption.

*Di-iodotyrosine.* Di-iodotyrosine has been reported by several workers to show thyroidal activity in tests on *Rana* and other tadpoles, but Morse [1914] noted that the activity was low as compared with dried thyroid, and Romeis [1923] found that several hundred times the dose of thyroxine was required. Gaddum [1927] emphasized that the substance produces effects on tadpoles only when given in large amounts over long periods, and found that by 1-day administration it was less than one-thousandth as active as thyroxine.

*l*(+)-Di-iodotyrosine, prepared from natural *l*(-)-tyrosine, used by us was definitely active on *Xenopus* tadpoles in our standard 7-day test with a 3-day dosage period. Our dosage period was longer than Gaddum's, but the amount necessary was still large. Doses of 20, 8 and 4 mg. in beakers, containing one tadpole each, gave 50, 40 and 0 % positive responses respectively. There was some change in shape of the tadpoles getting 4 mg. Under similar conditions of temperature and administration, 0.1 mg. of thyroxine gave a 60 % response, so that di-iodotyrosine may be considered to be 1/100 to 1/200 as active. The activity of di-iodotyrosine has a distinct bearing on the specificity of the test. If the whole of the acid-soluble organic iodine is present as di-iodotyrosine, the artificially iodinated proteins will contain anything up to 10 % of di-iodotyrosine, which, if active in protein linkage, would confer activity equal to that given by at most 0.1 % thyroxine. When the dose of iodinated protein is 1 mg. the di-iodotyrosine present might then have activity equal to that of 1  $\mu$ g. of thyroxine. Since 1 mg. of a good iodo-protein has activity equal to that of about 0.1 mg. of thyroxine, the di-iodotyrosine content cannot account for more than 1 % of its activity, which is negligible in relation to the accuracy of a biological test.

We have no evidence as to whether *l*(+)-di-iodotyrosine has direct thyroidal activity on tadpoles or whether the results observed are due to indirect action such as facilitating thyroxine synthesis by the tadpole. There is some doubt as to whether even very large doses of di-iodotyrosine show thyroidal activity in mammals [Gaddum, 1930; Thompson, Alper, Thompson & Dickie, 1934], and it is possible that the tadpole test is less specific than tests depending on changes in the B.M.R. of mammals. This possibility is also suggested by the reports that thyroxamine is inactive on mammals [Gaddum, 1930], but that di-iodotyramine is active on tadpoles [Abelin, 1919].

*Inorganic iodine.* A beaker dose of 10 mg. of potassium iodide failed to cause any obvious change in *Xenopus* tadpoles under the usual conditions of our test. This result is in keeping with those of previous workers who have investigated the effects of inorganic iodine. Similarly, prolonged feeding of tadpoles on sea-weed (*Laminaria*) ground to a fine powder did not expedite metamorphic changes.

*Dibromotyrosine and dichlorotyrosine.* Dibromotyrosine was reported by Abderhalden & Wertheimer [1928] not to show thyroidal activity in short duration tests

on tadpoles. Our results agree, since beaker doses of 10 mg. produced no effect in the usual 7-day test. Positive results were, however, obtained in longer tests with both the above compounds.

After a preliminary test to see in what strength of dibromotyrosine solution tadpoles would live, ten tadpoles about 20 mm. long were put into 2 l. of water with 200 mg. of dibromotyrosine and fed as usual on liver powder. The dose of dibromotyrosine was renewed weekly when the water was changed. After 19 days the nine surviving tadpoles were measured and put into two groups, A and B; five measuring 33, 33, 30, 26 and 24 mm. were replaced in dibromotyrosine solution (A), and four measuring 32, 28, 27 and 26 mm. were transferred to ordinary water (B). Control tadpoles (C) from the same batch averaged 36.4 mm. at this time, and were slightly better grown than in the two treated groups. This may partly have been due to their being less crowded. After 29 days in dibromotyrosine all tadpoles in group A had front legs, but although the average size was larger in the other two groups no legs had appeared. After 35 days all in group A were metamorphosing but none in group B or C, though two animals in the control group showed good growth of the back legs. After 42 days two animals were metamorphosing in the control group, but after 56 days three other controls had still not metamorphosed; those in group B metamorphosed slightly earlier. Some of the animals died just before the end of the experiment owing to an attack of fungus.

A second experiment was carried out along the same lines with six large tadpoles in 2 l. of water with 200 mg. of dibromotyrosine and six similar controls in another 2 l. jar. The water was not changed. There was no obvious difference between the tadpoles in the two jars during the 10 or 12 days, but the first tadpole metamorphosed in the experimental jar after about 17 days, and two others were metamorphosing 2 days later. The remainder metamorphosed in the next few days. In the control jar no front legs had appeared and there were no signs of metamorphosis, but in three other untreated animals of the same age metamorphosis was in progress. The control animals metamorphosed during the next 6 weeks; metamorphosis was not completed in the last one 2 months after the experimental ones had all changed.

A similar experiment was carried out with the same concentration of dichlorotyrosine. Five out of eight of these tadpoles had begun to metamorphose after 1 month in the solution when the controls were much less advanced. At that stage the experiment was terminated owing to an attack of fungus. In a repeat experiment with large tadpoles, metamorphosis was completed in the group treated with dichlorotyrosine when only one tadpole in the control jar showed front legs.

Considering these experiments as a whole, it appears that both the substances expedite metamorphosis in long-term experiments. This result is not unreasonable, since if tetrabromotyrosine [Gaddum, 1930] shows a fraction of the activity of thyroxine on mammals, dibromotyrosine might well show a fraction of the activity of di-iodotyrosine on tadpoles. There is, however, no evidence as to whether the effect is due to faint direct thyroidal activity or to some indirect stimulation of the thyroid or pituitary gland.

*Standard preparation for iodinated protein*

The results recorded in the present series of papers, like those of previous authors, show that the response of tadpoles to the same dose of the same thyroidal substance is influenced by a large number of variables, some of which cannot altogether be excluded by standardizing, so far as possible, the conditions of the test. The response will therefore vary from time to time and from place to place. Similar variation is, of course, found in all biological assay and is an irrefutable argument against any attempts to define activity in terms of an animal reaction. In conformity with this principle the activity of all the preparations discussed in the following papers is given in terms of two main preparations, iodinated caseins NC3 and NC4+5. The latter, which replaced the former, was considered preferable since it was carefully homogenized before samples were taken for iodine analysis and before its use as a laboratory standard.

In almost all cases where biological assay is required constantly, the problem of uncontrollable variations in response has been met by the establishment of a standard substance, in terms of which, by comparative assay, the activity of the unknown substance can be expressed. Most of the standards are on an international basis, the unit of activity being defined as the specific activity present in a given amount of the International Standard Preparation, which is usually held by, and dispensed from, some central source.

In the case of a crystalline characterized compound like thyroxine a standard preparation is not necessary, and dried thyroid preparations have been assayed, apparently successfully, by chemical estimation of thyroxine (acid-insoluble) iodine. With artificially iodinated preparations, however, the situation is different. The results recorded in a later paper in this series [Deanesly & Parkes, 1945] show that in our present state of knowledge the determination of the iodine content of these preparations is not a reliable guide to their biological activity. Biological assay is, therefore, necessary and is likely to be for an indefinite period, and all the considerations which have led to the establishment of standard preparations in the case of other biologically active substances seem to be operative in the case of iodinated proteins. It is one of the principles of biological assay that the standard must be administered in exactly the same way as the unknown substance under test. Thyroxine is, therefore, quite unsuitable as a biological standard of reference for iodinated proteins, since its availability to the organism is known to be quite different and its use would prohibit administration by mouth, which may well be desirable in tests for thyroidal activity of iodinated proteins on certain animals. The standard should, therefore, be an iodinated protein generally similar in nature to the substances likely to be tested against it. At present, the obvious choice is iodinated casein, about which a good deal is now known and which is likely to be the most important commercial product in the near future. It is not yet certain that other iodinated proteins, for instance, iodinated Ardcin or iodinated blood plasma, can safely be assayed against iodinated casein, but at present there is no reason to suppose otherwise. Certainly this problem will be no more difficult than similar ones which have constantly arisen in the use of other International Standards. and, in general, a standard for iodinated protein should prove easier to establish—

there is, for instance, no shortage of material—and not less effective in use than any of the existing International Standards.

*Statistical treatment of results*

The technique described above is a typical example of a biological assay method depending on a response which is qualitative for the individual but which becomes quantitative when the proportion of animals showing the all or nothing response is considered. Statistical methods applicable to the results of such tests are now well established. With a quantal response, the line relating the normal equivalent deviation to the logarithm of the dose is usually straight [Gaddum, 1933]. Minus quantities can be avoided by using probits.

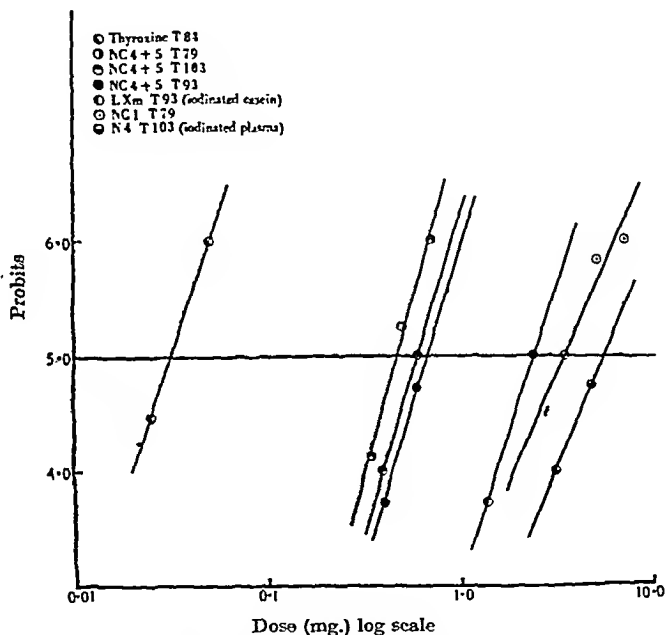


FIG. 3. Dose-response lines for thyroxine and iodinated proteins of different potencies, showing similarity of slope. T=test no.

In the case of the *Xenopus* experiments examination of the results for twenty-one tests, each comprising at least two points for each of two substances, led to the conclusions that

(a) the assumption of a linear relation between probit response and log dose was not disproved;

(b) in any one test the dose-response lines are parallel whatever the potency of the preparations (Fig. 3);

(c) the slope may vary from test to test, especially if conditions are changed (Fig. 4). Thus, in twelve consecutive tests, carried out at warm room temperature, the average slope was 3.24, significantly less steep than that of 5.95 for nine later tests carried out in an incubator. A slope approaching 6.0 may be regarded as very satisfactory.

If the potency of the standard in any one test is expressed as 1.0, and the potency of the unknown preparations in terms thereof, the limits of error ( $P=0.95$ ) in relation to the number of tadpoles used per substance, where the slope is not usually less than 5.0, will be as approximately as follows:

No. of tadpoles per substance	Limits of error ( $P=0.95$ )
20	0.66-1.51
30	0.72-1.40
40	0.75-1.34
80	0.81-1.23

Thus, with a test on thirty tadpoles per substance, if the unknown preparation has an apparent activity of 0.80 of that of the standard, the result is within the limits of error for  $P=0.95$ , and the difference from the standard is not significant. If, on the other hand, the apparent activity is 0.6 that of the standard, a real difference in potency can be assumed. Under the conditions of the test, therefore, and with the number of animals used, only wide differences between two preparations have reasonable significance, and it seemed unnecessary to calculate figures for the comparative activity of the substances used in every test. Where the points are suitably scattered, it is possible to fit a line graphically and obtain a figure approximating so closely to the calculated one that the estimate of the comparative potency of the preparation, or of the significance of its difference from the standard, is not materially affected. For use of the graphic method our aim has been to obtain both for the standard and for the unknown preparations a satisfactory distribution, above and below 50% response, of two or more points.

The comparative activity of two preparations or the comparative effectiveness of one preparation given by two different methods, as shown by the *Xenopus* tadpole test, has, therefore, been determined as follows. 'Sighting' tests are carried out to determine the appropriate dosage to give at least one point below and one above the 50% response level; where the slope of the dose-response line is as steep as in the *Xenopus* test, several initial experiments of this kind may be necessary. A definite test against the standard preparation is then carried out. The percentage response is converted into a probit, and plotted against dose on log paper, 0 and 100%

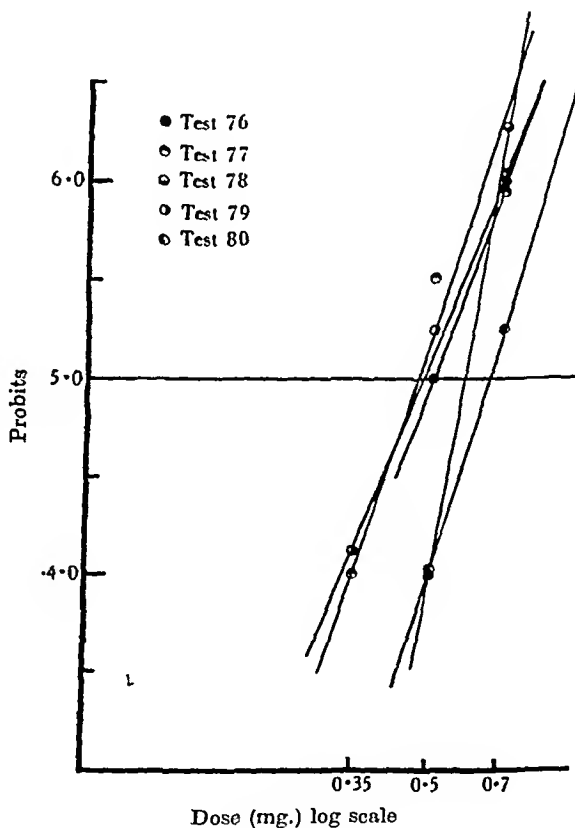


FIG. 4. Dose-response line for the laboratory standard iodinated casein (NC4+5) in five successive tests. M.E.D. varies from 0.46 to 0.66.

being given probits of 4.0 and 6.0 respectively where ten tadpoles are used in each group. A line is then fitted to the points by eye, and the dose corresponding to probit 5.0, 50 % response, determined. The activity of the standard is taken as 1.0, and the activity of the other substances tested at the same time is expressed in terms of this, less active substances having values below unity and more active ones above. The significance of the results is assessed by reference to the table given above.

#### SUMMARY

##### *The rearing of Xenopus tadpoles*

1. A study of the rearing of *Xenopus* tadpoles under laboratory conditions has been made to find out optimal conditions for obtaining large numbers of tadpoles for experimental purposes.
2. The conclusions of previous workers concerning the induction of oviposition and mating have been fully confirmed.
3. Factors affecting the growth of the tadpoles have been investigated in detail, growth being assessed by measurements of total length.
4. London tap water is a quite satisfactory medium if certain precautions are taken, and with a reasonable density of population and a proper regulation of the food supply the water need not be changed more often than every 2 or 3 weeks.
5. Powdered dried liver was found to be an excellent nutrient.
6. Combined feeding and density of population experiments showed that optimal growth was produced when the allowance of liver powder was 2-4 mg. per tadpole per day and the number of tadpoles did not exceed 8 per litre.
7. Round containers gave better results than rectangular ones, and deep ones better than shallow ones.
8. The size of the containers, within the limits tested, was not an important factor in growth.
9. Under good conditions, the tadpoles, and after metamorphosis the young toads, can be reared with little loss.

##### *Assay of thyroidal activity on Xenopus tadpoles*

1. The premature metamorphic changes induced in *Xenopus* tadpoles by exposure to thyroid preparations and related substances are described, and their suitability for assay purposes discussed.
2. The premature eruption of the front legs, which in *Xenopus*, unlike *Rana*, does not disturb the respiratory mechanism, appeared to be the most suitable criterion of thyroidal activity and has been used as the basis of an assay method.
3. The following variables have been considered in relation to the test: method of administration of active substance, concentration in relation to absolute amount of dose, duration of treatment, time of assessing response, size and age of tadpole, and temperature. Of these, temperature and duration of treatment are the most important factors.
4. In the method finally evolved, five tadpoles 18-25 mm. in length are placed in 200 ml. of water in a 250 ml. beaker, to which the given dose of the substance to be tested has been added as a fine suspension. After 3 days the tadpoles are changed

to tap water and the response is assessed, at the end of the seventh day, as the percentage of tadpoles showing eruption of one or both front legs.

5. Groups of ten or twenty tadpoles (two or four beakers) were used at each dose level, and the results examined by the statistical method usually applied to such quantal responses.

6. As with other biological assays, it is necessary to use a reference preparation, in terms of which the activity of unknown specimens can be expressed.

7. Dried thyroid powder, thyroxine, artificially iodinated proteins, and di-iodo-tyrosine are all active in this test. Inorganic iodine, dibromotyrosine and dichloro-tyrosine are inactive.

Our best thanks are due to Dr C. R. Harington, F.R.S., for his interest in the work and for supplying the tyrosine derivatives and the *l*(-)-thyroxine.

We are most grateful to Dr F. W. Landgrebe for valuable advice and for giving us the initial stock of toads. We are also indebted to Dr E. W. Hindle, F.R.S., for assistance at various points. The dried liver was kindly provided by Boots Pure Drug Co. The routine management of the stock was in the hands of our assistant, Miss Winifred Bailey.

Our grateful thanks are due to Mr K. L. Smith, Boots Pure Drug Co., who advised us constantly during the course of the experiments, formulated the general statistical conclusions recorded above, and generally gave us invaluable assistance with this aspect of the work. To Dr C. W. Emmens we are likewise indebted for most helpful advice in the quantitative presentation of the results.

We are also indebted to Messrs Boots Pure Drug Co. who supplied the dried thyroid and the iodinated caseins, to Mr G. Mooney, I.C.I. (Explosives) Ltd., who assisted with the work described on pp. 340-41, and to Mr A. P. Orr who supplied the seaweed meal.

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# THE PREPARATION AND BIOLOGICAL EFFECTS OF IODINATED PROTEINS

## 9. BIOLOGICAL ACTIVITY OF IODINATED PROTEINS

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(Received 13 October 1944)

The work on iodinated proteins, described in the present symposium of papers, was directed mainly towards large-scale experiments on the stimulation of lactation in cows. However, it became evident at an early stage that iodine analyses did not give a quantitative indication of the biological activity of the artificially iodinated proteins, and that tests on laboratory animals would be required before the main work on cows could be undertaken. In the first place, some use was made of the test based on changes in the oxygen consumption of guinea-pigs, determined by the method of Richards & Collison [1928]. This test is lengthy and laborious, and was of little value under the existing conditions for obtaining quantitative results on a large number of preparations. Other methods, reviewed in part by Burn [1937] and by Wokes [1935], were therefore considered. Determination of changes in the metabolic rate by estimation of the carbon dioxide output of mice by Mørch's method [1929] is not much more practicable on a large scale than the oxygen-consumption technique. The test depending on the loss of weight in guinea-pigs [Kreitmair, 1928] is known to give extremely uncertain results, an objection which also applies to the test based on increased resistance to acetonitrile [Hunt, 1905]. The restoration of growth in thyroidectomized rats by the administration of dried thyroid and iodinated proteins was investigated in detail by Rowlands [1945], who came to the conclusion that this reaction was not suitable for quantitative work. The use of the changes caused in the plumage of fowl by hyperthyroidism was considered but thought to be too speculative. Attention was then directed to the test based on the original observation of Gudernatsch [1914] depending on the induction of premature metamorphic changes in tadpoles. This method has been used with some success by several workers including Gaddum and Wokes, but, in this country, it has hitherto suffered from the extreme disadvantage that it could only be operated during a limited period of the year. The way in which this difficulty was surmounted has been described in a preceding paper in this series, and there is little doubt that it is now the method of choice.

The present paper records the results of the tadpole tests carried out on a large series of preparations of iodinated proteins including many of those which were used in the experiments on cows, and indicates (a) the extent to which activity in the tadpole test is prognostic of activity in stimulating milk yield, and (b) the difficulties in relating biological activity to iodine analysis.

## MATERIAL AND TECHNIQUE

The preparations considered are listed in Tables 1, 2 and 3 which give the iodine contents and the comparative biological activities of the iodinated plasmas, the iodinated Ardeins,\* and the iodinated caseins respectively. Almost all the individual preparations with the prefix N or L are fully described and their iodine analyses given in the paper by Pitt Rivers & Randall [1945]. Preparations not so described

Table 1. *Assay of iodinated plasma on Xenopus tadpoles*

Preparation	Iodine content (%)		Test no.	Reference substance	No. of tadpoles per sub-stance	Activity relative to reference substance	Average
	Total	Acid-insoluble					
N1	3.0	0.1	93	NC4+5	20	<0.05	<0.05
N4	5.4	0.4	103	"	20	0.11	0.11
			122	"	20	0.11	
LC	5.3	0.4	100	"	20	0.24	0.21
			110	"	20	0.17	
LB	3.01	0.51	109	"	25	<0.05	<0.05
LIII <sup>b</sup>	5.8	0.6	109	"	20	0.21	0.23
			122	"	20	0.25	
N2	5.6	0.7	97	"	20	0.22	0.23
			117	"	20	0.23	
LII	6.0	0.7	96	"	20	0.35	0.34
			117	"	20	0.33	
LIV <sup>a</sup>	7.94	0.84	100	"	20	0.08	0.08
			111	"	20	0.08	
LI <sup>a</sup>	5.75	0.9	100	"	10	0.40	0.48
			117	"	20	0.54	
			123	"	20	0.58	
			124	"	20	0.39	
LIV <sup>b</sup>	6.82	0.9	111	"	20	0.09	0.10
			100	"	20	0.11	
LIII <sup>a</sup>	5.9	1.18	97	"	20	0.30	0.31
			121	"	20	0.32	
LI <sup>b</sup>	5.3	1.5	97	"	30	0.41	0.40
			121	"	20	0.38	

include: iodinated plasma LC, a borate buffer preparation; iodinated plasma LB, prepared from whole blood; iodinated casein Ia, prepared according to the method of Ludwig & von Mutzenbecher [1939]; and iodinated casein Ib, prepared from Ia by incubation at 37°C. for 3 days in bicarbonate buffer. Four compound preparations were also examined: NCB1+2 was made up of 36 lb. of NCB1 and 38 lb. of NCB2, giving a theoretical average acid-insoluble iodine content of 1.64%. NC4+5 was compounded of 22½ lb. of NC4 and 30 lb. of NC5 and had an acid-insoluble iodine content of 1.6%. 50 lb. of this mixture, together with 16 lb. of NC7 gave NC4+5+7 with a theoretical acid-insoluble iodine content of 1.49%. A bulk mixture, totalling some 2000 lb. was also made of 60 iodinated casein preparations, NCB3 to NCB62. An aliquot mix of these 60 preparations was first made by mixing ½ of each batch. This aliquot had an acid-insoluble iodine content of 1.46%. Preparations with the prefix DT/S/- were prepared by I.C.I. (Explosives) Ltd. by a variety of methods.

\* Ardein is the registered trade mark for the ground-nut protein produced by I.C.I. (Explosives) Ltd.

Table 2. Assay of iodinated Ardein on *Xenopus tadpoles*

Preparation	Iodine content (%)		Test no.	Reference substance	No. of tadpoles per sub-stance	Activity relative to reference substance	Average
	Total	Acid-insoluble					
N5+6 MB	4.85	0.10	123	NC4+5	10	0.06	0.05
			126	"	20	0.04	
N9+10 MB	5.51	0.31	126	"	20	0.08	0.08
			63	NC3	20	0.34	0.42
DT/S/S00	3.8	0.4	127	NC4+5	20	0.50	
			130	"	20	0.10	0.15
N3SF	5.4	0.50	133	"	20	0.17	
			124	"	20	0.19	
N4SF	3.61	0.50	76	"	20	0.26	0.29
			78	"	30	0.32	
LXm	6.22	0.66	93	"	20	0.21	0.20
			95	"	20	0.18	
N4MB	5.76	0.88	96	"	20	0.45	0.46
			78	"	30	0.46	
LI $\alpha$	6.95	1.15	112	"	20	0.65	0.68
			121	"	20	0.70	
DT/S/824 (PB7)	7.2	1.2	63	NC3	20	0.34	0.42
			127	NC4+5	20	0.50	
DT/S/793 (SI/C)	6.9	1.2	17	NC3	30	0.18	0.29
			132	NC4+5	20	0.26	
			131	"	20	0.44	
DT/S/822 (PB5)	7.3	1.3	63	NC3	20	0.28	0.35
			127	NC4+5	20	0.42	
DT/S/834 (PB13)	7.6	1.6	128	"	20	1.20	1.43
			131	"	20	1.65	

The iodine analyses of the L and NC preparations were carried out by Mrs Pitt Rivers, of the NCB preparations by Messrs Boots Pure Drug Co., and of the DT/S/-preparations by I.C.I. (Explosives) Ltd. Pitt Rivers & Randall [1945] refer to certain anomalous results obtained in the iodine analyses. Samples of NC4 and NC5, for instance, both showed a considerably higher acid-insoluble iodine content than did the mixture NC4+5 made of other portions of the two preparations. Repeat analyses were confirmatory and the discrepancy was undoubtedly due to imperfect sampling of NC4 and NC5 which were not homogenized before the samples were removed for iodine estimations. In all cases, however, where error could have arisen from this cause, the biological and chemical assays were carried out on the same sample. Later preparations were homogenized before samples were taken. NC4+5 in particular was very carefully homogenized, and iodine analyses of the mixture carried out on different samples in three different laboratories gave identical results. For this reason NC4+5 was used as the reference preparation in all the later biological assays.

The biological activity of the preparations was examined by the *Xenopus* tadpole and the *Rana* tadpole techniques described in previous papers [Deanesly & Parkes, 1945; Deanesly, Emmett & Parkes, 1945]. NC3, and afterwards NC4+5, were used as reference preparations, in terms of which the activity of the other preparations was estimated as described in the assay paper. The activities of the two reference

Table 3. Assay of iodinated casein on *Xenopus tadpoles*

Preparation	Iodine content (%)		Test no.	Reference substance	No. of tadpoles per sub-stance	Activity relative to reference substance	Average
	Total	Acid-insoluble					
NCB10	7.66	0.85	124	NC4+5	20	0.76	0.71
			130	"	20	0.65	
NC1	6.3	1.0	77	"	30	0.09	0.11
			79	"	30	0.14	
			73	"	10	0.10	
NC7	7.02	1.18	67	NC3	20	0.66	0.70
			68	"	20	0.71	
			80	NC4+5	40	0.72	
NC2	7.3	1.2	69	NC3	20	0.78	0.69
			77	NC4+5	20	0.78	
			79	"	20	0.59	
			85	"	20	0.65	
			88	"	30	0.67	
NCB3/62 aliquot	8.02	1.46	104	"	20	0.64	0.77
			95	"	20	1.0	
			109	"	20	0.66	
NC4+5+7	7.91	1.49	103	"	20	0.84	0.89
			88	"	40	0.95	
			108	"	20	0.87	
NC6	7.28	1.55	66	NC3	20	1.0	0.76
			68	"	20	0.55	
			71	"	30	0.91	
			80	"	40	0.58	
NCB1+2	7.74	1.64	83	NC4+5	30	0.89	0.89
			108	"	20	0.93	
			103	"	20	0.84	
LXm	11.1	2.0	93	"	20	0.27	0.26
			95	"	20	0.24	
NC5	8.25	2.0	66	NC3	20	1.2	0.94
			68	"	20	0.67	
			71	"	30	0.82	
			80	NC4+5	10	1.1	
LIa	8.7	2.05	130	"	20	0.57	0.59
			132	"	20	0.60	
DT/S/823 (PB6)	7.4	2.3	63	NC3	20	0.23	0.25
			131	NC4+5	20	0.33	
			134	"	20	0.20	
LIb	9.25	2.45	114	"	20	0.85	0.81
			124	"	20	0.76	
NC4	8.8	2.6	69	NC3	20	1.1	1.17
			71	"	30	1.3	
			77	NC4+5	20	1.1	
NC3	9.0	2.7	72	"	30	0.84	0.95
			104	"	20	0.95	
			108	"	20	1.03	
			112	"	20	0.93	
NCB36	8.62	2.88	124	"	20	1.1	1.0
			130	"	20	0.90	
DT/S/829 (PB11)	8.9	3.3	67	NC3	20	0.39	0.31
			129	NC4+5	20	0.26	
			132	"	20	0.29	
LGEC	10.8	3.37	104	"	20	0.22	0.22

preparations assayed on *Xenopus* tadpoles were indistinguishable (Table 3), and they have been considered to be identical for the purpose of calculating, for other preparations, the average activity obtained in different tests. The M.E.D. 50% positive response for these two preparations under the conditions of the test was usually between 0.4 and 0.6 mg.

#### COMPARATIVE ACTIVITY OF VARIOUS IODINATED PROTEINS

Tables 1, 2 and 3 give most of the information available about the forty-two preparations of iodinated proteins (twelve plasmas, twelve Ardeins, and eighteen caseins), in addition to the reference preparation NC4+5, which were investigated. A large number of 'sighting' tests were, however, carried out in addition to the actual assays listed in the tables, and for some of the preparations confirmatory results were obtained on *Rana* tadpoles. The following notes are in amplification and explanation of the tables.

*Iodinated plasma.* N1 failed to produce a positive response in a dose of 8 mg. in a test in which 0.4 and 0.6 mg. of NC4+5 gave 10 and 40% positive responses. Its activity would appear, therefore, to be less than 0.05 that of the reference substance. The preparation, however, caused some change in shape of the tadpoles and the appearance of unextruded limb buds and seemed, therefore, to have a trace of activity. Similar results were obtained with LB. Positive responses were obtained with N4, LIVa and LIVb, but large doses, up to 8.0 mg. were required, and the activity was definitely low. This result is surprising in the case of the last two because of their comparatively high iodine content, but duplicate assays gave concordant results. None of the other plasmas calls for special mention. The best of these plasma preparations was only about one-half as active as the reference preparations of iodinated casein.

A few of the iodinated plasmas were tested on *Rana* tadpoles by the 1944 method. LA evoked very little response and appeared to have little activity. LIIIb and Ib showed equal activity, about one-third that of the reference substance NC4+5.

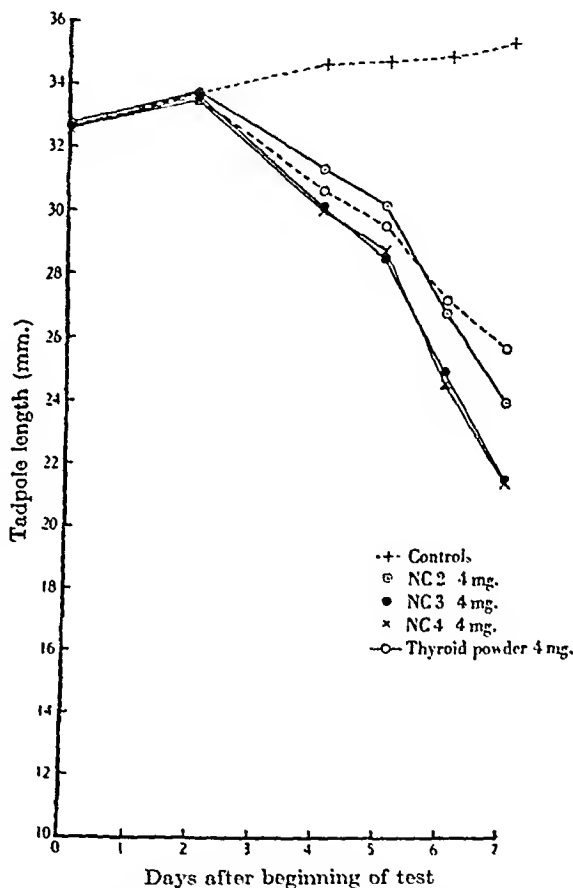


FIG. 1. Comparative activity, shown on *Rana* tadpoles by the 1943 method, of NC2, NC3 and NC4, and of dried thyroid. NC3 and NC4 are equally active; NC2 is slightly less active and about equal to the thyroid preparation. Ten tadpoles in each group.

These results, so far as they go, are concordant with the much more extensive tests on *Xenopus* tadpoles.

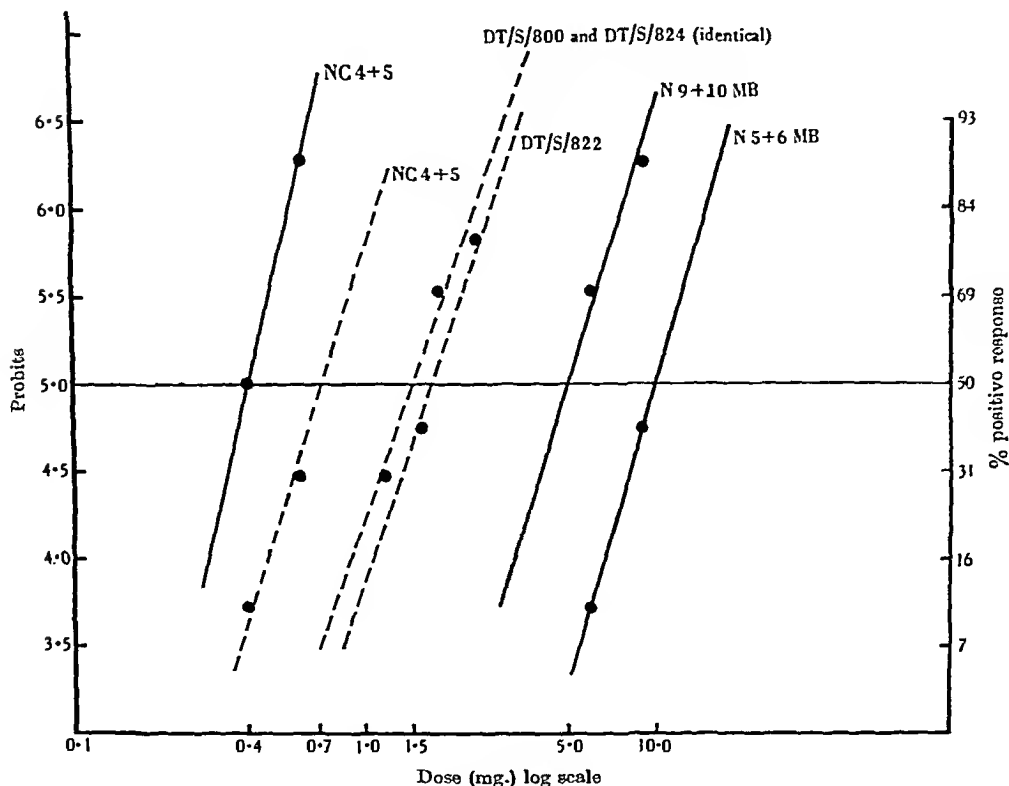


FIG. 2. Results of two different tests on *Xenopus* tadpoles. —=test 126; ----=test 127. NC4+5=iodinated casein standard. Other preparations all iodinated Ardeins. Results are as follows:

	Preparation	M.E.D.	Potency
Test 126	NC4+5	0.40	1.0
	N9+10MB	5.2	0.03
	N5+6MB	10.0	0.04
Test 127	NC4+5	0.75	1.0
	DT/S/800	1.5	0.50
	DT/S/824	1.5	0.50
	DT/S/822	1.8	0.42

As indicated by the M.E.D.'s for the standard substance the tadpoles used in test 126 were more sensitive and those used in test 127 less sensitive than usual.

*Iodinated Ardein.* The twelve preparations examined ranged from highly active to virtually inactive, the majority being of rather low activity compared with most of the iodinated caseins. Both N5+6MB and N9+10MB evoked definite positive responses in high dosage (6–9 mg.), but the activity was less than one-tenth that of the reference substance (Fig. 2). The other preparations call for no special mention, except DT/S/834 (PB13) iodinated Ardein prepared by the same method as the NC

iodinated casein series of Pitt Rivers & Randall, which had high activity apparently rather greater than that of NC4+5.

Some of the iodinated Ardeins were tested on *Rana* tadpoles. By the 1943 method of relatively long dosage at room temperature N4SF was about one-half as active as N4MB which, in turn, had about one-half the activity of iodinated casein NC3. N9+10MB was inactive in a dose eight times that required of NC3. By the 1944 method of short dosage at a higher temperature, a more sensitive test, LGEC showed little activity, N9+10MB was less than one-tenth and LXm less than one-quarter as active as NC4+5. DT/S/834 was considerably more active. These results are quite concordant with those obtained on *Xenopus* tadpoles.

*Iodinated casein.* Eighteen preparations in all were examined. Of these, seven had activity not very different from NC4+5, six were apparently of rather less activity, four were only about one-quarter and one only about one-tenth as active as the standard. For fifteen of the preparations successive tests gave satisfactorily concordant results. For the other three, NCB3/62, NC6 and NC5, results were somewhat variable, but not more so than might be expected occasionally from the statistical considerations set out previously [Deanesly & Parkes, 1945]. NC2-7 and the NCB series had activity similar to or rather greater than that of the ordinary commercial preparation of dried thyroid.

Some of the iodinated caseins were tested on *Rana* tadpoles. By the 1943 method, NC2 was more than four times as active as NC1, and NC3 and NC4 were equally active and produced a greater response than NC2 at the same dose level (Fig. 1). By the 1944 method, NC1 was about one-tenth, NC2 and NCB3/62 aliquot about three-quarters, and NCB1+2 about equally as active as the standard. NCB10 was slightly less active than NCB36. All these results are concordant with those obtained on *Xenopus*.

#### ACTIVITY ON TADPOLES RELATIVE TO ACTIVITY ON COWS

The following preparations, of those listed in Tables 1, 2 and 3, have been tested for their capacity to stimulate established lactation in cows.

Iodinated plasma: N4.

Iodinated Ardein: LXm, N4MB, and N9+10MB.

Iodinated casein: NC1, NC2, NC3, NCB1+2, LXm, NC4+5+7, and NCB3/62.

The results so far reported by Blaxter [1945 *a, b*] relate to the one plasma, the three Ardeins, and the first four of the caseins. The eight preparations tested on both tadpoles and cows vary greatly in activity and include three different proteins iodinated by various methods, and they give a most useful indication of the extent to which activity on tadpoles is prognostic of activity on cows. Apart from its practical importance this point is of considerable interest in that among the many and varied types of biological response to thyroïdal activity it is difficult to think of two more dissimilar ones than induction of premature metamorphosis in tadpoles and the stimulation of lactation in cows. In general the comparative activities of different preparations with thyroïdal activity are similar in different types of test, but, even so, the correspondence between the tadpole and the cow responses was

surprisingly good. The results on cows with the eight preparations used on both species are given in Table 23 of Blaxter's paper [1945*b*] in which comparative activity is assessed by reference to NC3 (NC4+5 was not tested on cows). This information correlates with that given in Tables 1, 2 and 3 of the present paper as shown in Fig. 3. From this graph it appears that NC2 was rather more active on cows than would have been expected from the tadpole results, but allowing for the error of biological measurement in both species it is likely that there is complete correspondence between activity on tadpoles and activity on cows. The important practical conclusion follows that iodinated proteins intended for use on cows can safely be assayed on tadpoles without the trouble and expense of carrying out assays on cows.

#### RELATION BETWEEN BIOLOGICAL ACTIVITY AND ACID-INSOLUBLE IODINE CONTENT

The effect of the method of iodination on the iodine content of the treated protein has received considerable attention and is discussed in some detail by Pitt Rivers & Randall [1945]. The relation between the iodine content, particularly the acid-insoluble, the so-called thyroxine iodine content, and the thyroidal activity is also complex, and has received comparatively little attention except from Reineke & Turner [1942] who deal at some length with

the relation between total iodine and biological activity. They conclude that a total organic iodine content of about 6.5% is optimal for iodinated casein prepared by their method. There seems, however, to be no adequate information about the shape of the curve relating biological activity to acid-insoluble iodine content in different proteins iodinated by different methods. Some conclusions, however, about the relation between method of iodination, total and thyroxine iodine content, and biological activity can be drawn from the paper by Pitt Rivers & Randall considered in relation to the data given in Tables 1, 2 and 3 of the present paper. The preparations considered were not dialysed or otherwise treated to remove inorganic iodine before estimation, so that the total iodine figures are complicated by the inclusion of amounts, no doubt variable, of uncombined iodine. This complication is likely to mask any strict relation between total iodine and biological activity, and in the following discussion only acid-insoluble iodine is considered.

*Iodinated plasma.* The twelve preparations considered had acid-insoluble iodine contents varying from 0.1 to 1.5%; the biological activity varied from negligible to

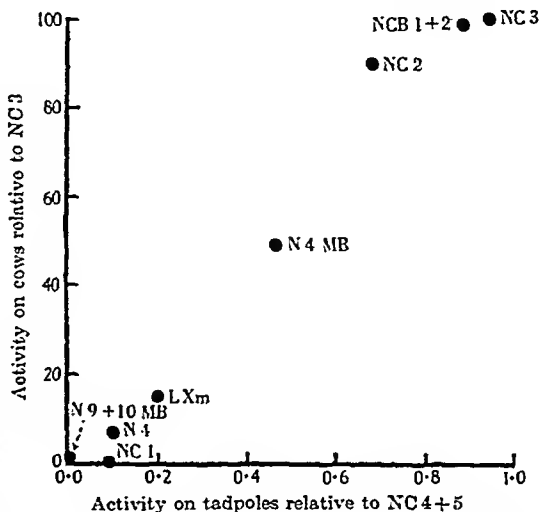


FIG. 3. Activity in causing premature metamorphosis in *Xenopus* tadpoles relative to activity in stimulating milk yield in cows.

Iodinated plasma: N 4.

Iodinated Ardeins: LXm, N 9+10 MB, N 4 MB.

Iodinated caseins: NC 1, NC 2, NC 3, NCB 1+2.

Activity on cows from Blaxter [1945 *a*, *b*].



up to 0.48 that of the standard. The relation between the two is shown graphically in Fig. 4. It will be seen that there is a general tendency for activity to increase with iodine content, but that there is great variation at any particular level of iodine content. This variation is of a magnitude which makes it unlikely to be an effect of the combined errors in biological and chemical assay. Thus, *LIa*, *LIVb* and *LIVa*, having acid insoluble iodine values of about 0.9%, varied in activity from 0.08 to 0.48 that of the standard. All three of these are out of the general run of the preparations. Four separate assays were carried out on *LIa*, and there is little doubt that it is the most active of the plasma preparations examined. The assays on *LIVa* and *LIVb* seemed unexceptionable and their low activity is not in doubt. There is no obvious explanation of this finding. As regards the rest of the plasma

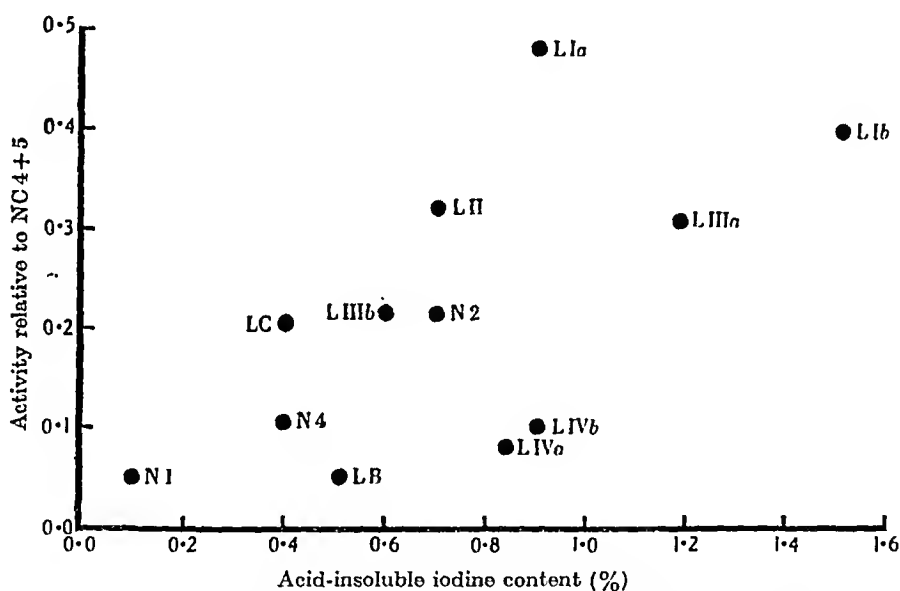


FIG. 4. Iodinated plasmas. Relation between biological activity on tadpoles and acid-insoluble iodine content.

preparations little can be said, except that those with the higher acid-insoluble iodine content are, on the whole, the more active. It may be significant that N1, N2 and N4, all large-scale preparations made by the same method, show good gradation of biological activity according to acid-insoluble iodine content.

*Iodinated Ardein.* The twelve preparations examined had acid-insoluble iodine contents varying from 0.1 to 1.6% and biological activities varying from 0.05 to 1.43 that of the standard. The last figure, however, was quite exceptional, the next highest being 0.68 obtained for *LIa*, a preparation made from purified Ardein with a high content of tyrosine. It will be seen from Fig. 5 that, as with the plasmas, there is a general tendency for biological activity to increase with acid-insoluble iodine content, but that there are outstanding anomalies. These, however, are all among the DT/S/- series and if this series is omitted the remaining seven preparations show a good correlation between biological activity and acid-insoluble iodine content. If the two salt fraction (SF) preparations, which probably consisted mainly

of iodinated globulin, are also omitted, the remaining five preparations, which were all made by essentially the same technique, show excellent correspondence between the two indices. The results with these five preparations suggest that by the method of iodination employed comparatively little thyroidal activity is conferred on Ardein by the combination of acid-insoluble iodine up to 0.7%. Quantities above this up to 1.2% result in thyroidal activity similar to that shown by commercial dried thyroid powder containing 0.09–0.11% acid-insoluble iodine. In other words, ten

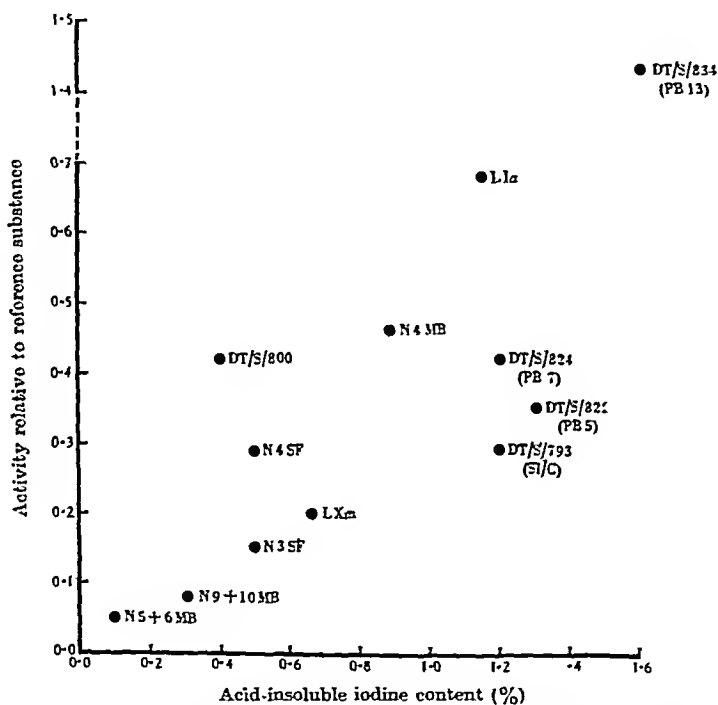


FIG. 5. Iodinated Ardeins. Relation between biological activity on tadpoles and acid-insoluble iodine content.

times the amount of acid-insoluble iodine is required to make iodinated Ardein equally as active as dried thyroid.

The DT/S/- series are most irregular. DT/S/800, with a low acid-insoluble iodine content, was fairly active, more active in fact than DT/S/793 and DT/S/822 with three times the iodine content. DT/S/834, by contrast, with 1.6% acid-insoluble iodine, was the most active iodinated protein examined, being considerably better than the iodinated casein standard. The reason for the low activity of the preparations with 1.2–1.3% acid-insoluble iodine probably lies in the method of preparation, but the very high activity of the last preparation is curious since it was prepared by the same method as the casein standard. This preparation is of especial interest in showing the potentialities of a vegetable protein for the artificial production of substances with thyroidal activity.

*Iodinated casein.* The eighteen preparations examined had acid-insoluble iodine contents from 0.85 % to nearly 3.4 %, i.e. nearly all the caseins had a higher acid-insoluble iodine content than almost all the plasmas and Ardeins. This fact does not necessarily imply that casein is more suitable for iodination, since, among the preparations made in London and Nottingham, high temperature incubation was not employed for the plasmas and Ardeins, only for the caseins NC2-7 and the NCB series. There is no information, for instance, as to the effect of incubation at 70°C. on the potency of iodinated plasmas. The absence of caseins with a low iodine

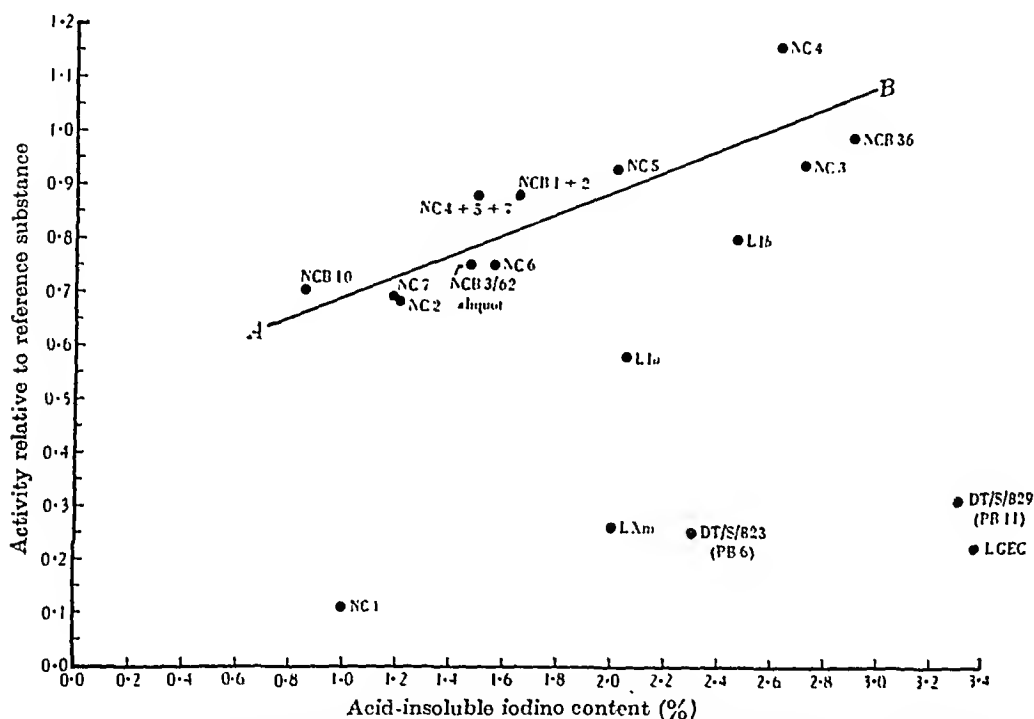


FIG. 6. Iodinated caseins. Relation between biological activity on tadpoles and acid-insoluble iodine content. The line *AB* gives a visual indication of the relationship in NC2-7 and the NCB series. Over the range covered by this line and with these preparations, an increase of 1 % in acid-insoluble iodine gives an increase in activity equal to 0.2 that of the standard. As the latter had activity roughly equal to that of dried thyroid containing 0.1 % acid-insoluble iodine, it may be concluded that only 2 % or less of the acid-insoluble iodine above 1.0 % in the NC2-7 and NCB preparations is thyroxine iodine.

content makes it difficult to compare the initiation of thyroidal activity in this protein with that in plasma and Ardein. The biological activity of the iodinated caseins varied from negligible to very good. Fig. 6 shows that the preparations fall into two groups, a low activity group with acid-insoluble iodine content between 1.0 and 3.4 %, and a moderate or high activity group with acid-insoluble iodine content of about the same range. The five preparations in the low activity group include LGEC which was purposely over-iodinated, LXm the low activity of which is not easy to account for, NC1 which was made by an untried method, and two DT/S/- preparations. The thirteen preparations in the high activity group include NC2-7 and the NCB series, and two L preparations. The latter fall slightly apart from the former and were made by a different method (see p. 357). NC2-7 and the

NCB series, all made by a method involving incubation at 70°C., show a gradually increasing activity with increase of acid-insoluble iodine; even allowing for the error of the biological test, the general tendency is unmistakable. The increase, however, is at best only about 50%, whereas the increase in acid-insoluble iodine is from 0.85 to 2.84%, more than 300%. It is obvious from the diagram that for this relationship to be extrapolated backwards would involve considerable activity on the part of untreated casein. In other words, the relationship between acid-insoluble iodine content and biological activity changes as casein is progressively iodinated and has at least two phases. The first phase ends sometime before the acid-insoluble iodine reaches 0.8% and is one of comparatively rapid increase of biological activity with increase of acid-insoluble iodine. The second phase begins when the acid-insoluble iodine is not more than 0.8% and is one of slow increase of biological activity with increase of acid-insoluble iodine. By analogy with the results on plasma and Ardein it may be suggested that the first of these phases itself consists of two parts, the first being of slow increase of biological activity and the second of very rapid increase. If so, the whole curve relating biological activity to acid-insoluble iodine would be sigmoid in nature (at least up to an acid-insoluble iodine content of 3.0%) though at no point is the combination of iodine as effective as is seen in the case of the thyroid gland. This generalization, of course, relates only to preparations made by the method employed for NC2-7 and the NCB series, and a quite different relationship may be found in series of compounds made by other methods.

#### DISCUSSION

Reineke & Turner [1942] conclude that the total organic iodine figure for iodinated casein gives a good indication of biological activity, the optimum figure being about 6.5%. Appreciable rise above this, or fall below, both result in lower biological activity. These authors appear, however, to find some significance in the fact that the biological activities of iodinated casein and of thyroxine are about the same in relation to their organic iodine content. This conclusion apparently overlooks the facts that (a) thyroxine in isolated form is known to be less effective than thyroxine in protein combination, and (b) much of the organic iodine in iodinated casein is acid-soluble and cannot, therefore, be thyroxine iodine. This acid-soluble iodine, contained probably in di-iodotyrosine, is unlikely to add appreciably to the biological activity of iodinated proteins even in the tadpole test [Deanesly & Parkes, 1945].

In all the work described in the present symposium of papers acid-insoluble iodine has been taken as the initial criterion of whether a particular preparation was likely to be active. Clearly, however, in the artificially iodinated proteins, total acid-insoluble iodine is only a very rough guide to biological activity, and it seems that a sharp distinction must be drawn between thyroxine iodine and non-thyroxine acid-insoluble iodine. It has been emphasized above that a good specimen of iodinated protein, containing perhaps 1.0-2.0% of acid-insoluble iodine, is but little more active in the tadpole test than the ordinary commercial specimen of dried thyroid gland containing about 0.1%. This discrepancy is not a peculiarity of the tadpole test, since dried thyroid and artificially iodinated proteins also show the same order of biological activity in other tests on other animals, viz. inducing growth in thyroid-ectomized rats [Rowlands, 1945]; stimulating milk yield in cows (comparison of the

data of Graham [1934] and Blaxter [1945b]); and raising the B.M.R. in guinea-pigs [Folley & Emmett, private communication]. Several different explanations might be offered of this interesting discrepancy: (a) the thyroxine in the artificially iodinated protein might be less available or less useful to the animal than that in thyroid tissue; (b) antagonistic substances might be present in the artificially iodinated substances; (c) the activity of artificially iodinated proteins might depend wholly or partly on the presence of some compound less active than thyroxine; and (d) the active principle might be thyroxine only, the greater part of the acid-insoluble iodine being non-thyroxine iodine.

Explanation (a) is not likely. It is well known that, even allowing for the probable inactivity of the *d*-form, isolated thyroxine is not so efficient biologically as thyroxine combined with thyroid protein, probably because of its lesser availability to the animal. In the test on *Xenopus* tadpoles [Deanesly & Parkes, 1945] for instance, 0.05 mg. of thyroxine gives about the same response as 0.5 mg. of dried thyroid containing a maximum of 0.15 % thyroxine, i.e. the combined thyroxine is seventy times as effective as the hormone deprived of its protein combination. There is, however, no clear evidence that the thyroxine contained in iodinated casein is less effective than the thyroxine present in thyroglobulin, and it is difficult to believe that ineffectiveness due to this cause would appear consistently in the several types of test enumerated above.

Explanation (b) is not supported by any evidence, as yet, of the presence in artificially iodinated protein of substances antagonistic to thyroidal action.

Explanation (c) appears to have been disproved by the isolation of thyroxine from iodinated protein, first by Ludwig & von Mutzenbecher [1939] and later by Reineke & Turner [1942], in amounts more than accounting for its biological activity.

Explanation (d) is much the most likely. It has been pointed out by Pitt Rivers & Randall [1945] that the iodination of the tyrosine constituent of the casein, and the oxidation of two molecules of di-iodotyrosine thus formed to give thyroxine is likely to result, under relatively crude conditions, in the formation of biologically inactive by-products containing acid-insoluble iodine. On this hypothesis it is perhaps surprising that as little as 5 % of the acid-insoluble iodine should be thyroxine iodine, but the idea makes more understandable the fact that in the NC and NCB series of preparations described by Pitt Rivers & Randall, manufactured under closely similar conditions, the acid-insoluble iodine varies from 0.8 to 2.9 % without, apparently, variation of similar magnitude in the biological activity of the preparations. If, as suggested above for the NC and NCB preparations, the curve relating biological activity and acid-insoluble iodine is triphasic in nature, it may be postulated that the first iodine combined in acid-insoluble form, up to perhaps 0.2–0.4 %, produces little thyroxine, the next, up to perhaps 0.8 %, produces considerable thyroxine, and that further combination of iodine gives little thyroxine and a high proportion of inactive substance containing acid-insoluble iodine. Whatever the exact nature of the relation in different series of preparations, it must be an expression of chemical reaction involving the iodination of tyrosine to di-iodotyrosine and the oxidation of two molecules of the latter to form thyroxine, both stages of this process, as well as the formation of inactive by-products, being affected by the amount of iodine added and the conditions employed. The relative amounts of thyroxine iodine

and non-thyroxine acid-insoluble iodine found at any particular level of total acid-insoluble iodine will thus be much influenced by the method of preparation. Over-iodination, for instance, produces low activity preparations, probably because the over-oxidation of di-iodotyrosine produces less thyroxine and more inactive compounds containing acid-insoluble iodine.

The work on artificially iodinated proteins raises the interesting speculation as to whether the acid-insoluble iodine of dried thyroid gland contains a non-thyroxine component. The accepted method [*British Pharmacopoeia*, 1932] of determining the biological potency of dried thyroid preparations is by the estimation of acid-insoluble iodine. This procedure was based on chemical considerations, and assumed that thyroxine was the only source of acid-insoluble iodine or at least contributed a constant proportion of the whole. Some doubts, however, have been expressed as to whether biological activity can accurately be determined even in the case of thyroid preparation by determination of the amount of acid-insoluble iodine. Thus, Gaddum & Hetherington [1931] came to the conclusion that the so-called thyroxine iodine content did not give a reliable indication of the capacity of thyroid preparations to raise the B.M.R. of mice as determined by carbon dioxide output. In the tadpole metamorphosis test Wokes [1938] and Dutt & Mukerji [1942] came to the conclusion that the figures for the total iodine content of different dried thyroid preparations agreed better than did those for thyroxine iodine with the observed biological activity of the preparations. Wokes emphasizes, however, that neither figure agreed very well. In conclusion, it can only be said that much further work is required to determine accurately the relation between biological activity and acid-insoluble iodine of artificially iodinated preparations, the factors which influence the relation, and the nature of the presumed inactive substances containing acid-insoluble iodine. Further investigation of dried thyroid preparations of differing iodine contents would also seem promising.

#### SUMMARY

1. Twelve preparations of iodinated ox blood plasma, twelve of iodinated groundnut protein (Ardein), and eighteen of iodinated casein have been examined for biological activity in the tadpole metamorphosis test.

2. The activity varied from negligible to greater than that of the ordinary commercial specimen of dried thyroid gland.

3. In eight preparations tested on both species relative capacity to induce premature metamorphosis in tadpoles corresponded closely with relative capacity to stimulate milk yield in cows.

4. The relationship between biological activity and acid-insoluble iodine is influenced by the method of iodination and probably by the type of protein iodinated. With casein preparations iodinated by the same method the relationship is certainly not linear and is probably sigmoid.

5. With the best artificially iodinated preparations so far examined the acid-insoluble iodine content is more than ten times that of dried thyroid preparations showing the same order of biological activity. This indicates that in the artificial preparations most of the acid-insoluble iodine is not thyroxine iodine and emphasizes

the need for work on the inactive substances contributing to the total acid-insoluble iodine.

We are most grateful to Dr C. R. Harington, F.R.S., and to Mrs R. V. Pitt Rivers for the specimens of the L series of preparations, for information about several of the preparations not recorded by Pitt Rivers & Randall [1945], and for their interest and advice.

Our best thanks are also due to Mr K. L. Blaxter, National Institute for Research in Dairying, with whom we have exchanged information at all stages of the investigation, to Miss J. Emmett who assisted with many of the assays, and to Dr S. J. Folley for the information recorded on p. 368.

The DT/S/- preparations were made by I.C.I. (Explosives) Ltd. and we are indebted to Dr D. Traill for permission to include the biological results and the iodine analyses in the present paper.

The N preparations were made by Messrs Boots Pure Drug Co. Ltd., for the Agricultural Research Council, as recorded by Pitt Rivers & Randall [1945].

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# ENTEROHEPATIC CIRCULATION OF OESTROGENS

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(Received 5 December 1944)

Evidence that the liver destroys the activity of oestrogens has been strongly presented in a long list of publications and there appears to be no serious dissenting opinion [Cantarow, Rakoff, Paschkis, Hansen & Walkling, 1943]. The inactivation of oestrogens by isolated liver tissue is one of the clearest demonstrations of this hepatic function [Silberstein, Engel & Molnar, 1933; Heller, 1940; Engel, 1941]. It should also be recalled that Golden & Sevringhaus [1938] implanted ovaries in the mesenteries of rats without producing oestrus; that Biskind [1941] failed to obtain atrophy of the reproductive organs of male rats when pellets of oestrone were implanted into the spleen but that atrophy resulted when the implants were made subcutaneously; and that damage to the liver favours higher concentrations of oestrogens in blood [Talbot, 1939; Pincus & Martin, 1940] and in urine [Glass, Edmondson & Soll, 1940]. Cantarow, Rakoff, Paschkis & Hansen [1942] gave evidence for 'the existence of an enterohepatic circulation of oestrogens, similar to that of bile acids'. Such a mechanism might account for some of the differences noted between stilboestrol and other oestrogens. It also suggests that some of these differences, i.e. the more potent [Matthews, Schwabe & Emery, 1942] and longer action of stilboestrol [Emery, Matthews & Schwabe, 1942], might disappear if the enterohepatic circulation was eliminated. In this report we present data describing the oestrogenic effects of stilboestrol and oestrone in rats whose enterohepatic circulation was interrupted by severing their bile ducts.

## MATERIAL AND METHODS

Adult albino rats were used. The skin of the lumbar region was shaved and sterilized with alcohol and the ovaries removed through a mid-line incision. After recovery many of these animals had a second operation. The skin of the abdomen over the liver was likewise shaved and sterilized; a skin incision was made over the stomach and the bile duct near the duodenum was exposed and severed between double ligatures. A few days after the second operation the animals were weighed and injected. Some died within a few days and others died following the injections, but as a whole the death-rate throughout the experiment was not high. Some of the rats appeared in good condition even 4 months after the bile ducts were severed. Success of the operation was evident by clay-coloured stools and icteric urine. The controls were similarly oophorectomized, but a second operation or laparotomy was not performed.

The oestrogens used were oestrone and stilboestrol. The dosage was 1 mg. dissolved in peanut oil given at a single injection either intraperitoneally or subcutaneously. Oestrus was determined daily by vaginal lavage as previously described [Emery & Schwabe, 1936]. The experimental and control groups were under



observation simultaneously and in small groups, thus eliminating possible errors in technique and seasonal variations. The rats were kept in a constant-temperature room and fed dog chow and water.

### RESULTS

The durations of oestrus following injections of 1 mg. of oestrone or stilboestrol are summarized in Table 1. Probably the most interesting feature of this table is the mean duration of oestrus in rats with severed bile ducts. It will be seen by reference to groups A and B that following intraperitoneal injections of oestrone the mean duration of oestrus was 6.0 days in the experimental rats and 5.4 days in the controls. These figures are nearly alike, but the larger of the two was obtained in rats with the bile ducts cut. Further confirmation of this was obtained when the injections were made subcutaneously. Here mean values of 15.5 and 11.2 days were obtained, a difference of 38.4% between the experimental and control rats (groups C and D).

Table 1. *Duration of oestrus in days following single injections of oestrogens*

Group	No. of tests	Injection†	Duration of oestrus (days)			Significant ratio‡	Coefficient of variation‡
			Mean	Median	Range		
1 mg. of oestrone in peanut oil							
A*	22	Ip.	6.0	5.55	3-12	0.8	37.0
B	20	Ip.	5.4	5.17	3-12		50.0
C*	20	Sq.	15.5	15.50	9-24	3.1	20.7
D	17	Sq.	11.2	11.25	5-19		39.2
1 mg. of stilboestrol in peanut oil							
E*	21	Ip.	9.0	9.75	4-19	1.4	44.5
F	21	Ip.	7.2	9.5	4-15		53.1
G*	24	Sq.	17.5	17.5	8-28	3.5	29.0
H	23	Sq.	12.9	13.0	4-23		31.2

\* Experimental groups with severed bile ducts (62 rats). There were 46 control rats.

† Ip.=intraperitoneal; Sq.=subcutaneous.

‡ S.D.=standard deviation= $\sqrt{(\sum d^2)/n}$ . Coefficient of variation=S.D.  $\times$  100/mean. Standard error of difference= $\sqrt{[(S.E._1)^2 + (S.E._2)^2]}$ . S.E.=standard error of mean=S.D./ $\sqrt{N}$ . Significant ratio=mean difference divided by the standard error of difference.

The validity of these results obtained with oestrone is further confirmed by similar experiments with stilboestrol. An equal amount by weight of stilboestrol is known to induce a more prolonged period of oestrus than that obtained with oestrone [Emery *et al.* 1942 and Table 1]. When stilboestrol was given intraperitoneally the oestrus that followed was only slightly longer in rats with bile ducts severed, the means being 9.0 and 7.2 days in the experimental and control rats respectively. Although the mean difference is only 2 days it is worth noting because here again oestrus is prolonged in the group with severed bile ducts. The effect is much more pronounced when the hormone is given subcutaneously; this is shown in groups G and H of Table 1. The mean difference is 4.6 days or a change of 36.5%. The 'significant ratio' is 3.5 and this further attests the significant difference between these two groups (Table 1). Thus we see that the action of stilboestrol is not only more prolonged than that of oestrone but that this effect is amplified in rats with severed bile ducts and especially noted following subcutaneous administration.

## DISCUSSION

Although the data at hand support the conclusion that ligation of the bile ducts in rats prolongs the duration of oestrogenic activity this does not appear to mean that these rats are more sensitive to oestrogens as determined by threshold dosage. Many tests were made with oestrone and stilboestrol given in subthreshold and greater doses, but no evidence was obtained that the rats with severed bile ducts were the more sensitive. When injected into the spleen *in situ* seventeen times as much stilboestrol is required to produce oestrus as when the injections are given into a subcutaneously transplanted spleen [Segaloff, 1944]. It would therefore seem that the livers of our rats were destroying oestrogens at a nearly normal rate and therefore the minimal dose in the experimental and control groups did not differ. Our previous difficulty in determining the minimal dose of stilboestrol [Matthews *et al.* 1942] makes one cautious and therefore further comment on this point is reserved.

In regard to the thesis that elimination of enterohepatic circulation would also eliminate at least part of the differences between the activities of oestrone and stilboestrol, it seems evident that if the enterohepatic circulation were the mechanism underlying the duration of oestrus, then when the bile duct was severed the duration of oestrus would be similar in length for either oestrone or stilboestrol. But, as we have pointed out, this did not happen. The reason that stilboestrol is more active than oestrone cannot, therefore, be explained by assuming an enterohepatic circulation for stilboestrol. Oestrone, although excreted with bile, would be largely destroyed in the intestines and so would not be absorbed. As shown in Table 1 the effect of 1 mg. of oestrone given intraperitoneally is entirely gone in approximately 5 days, and by similar comparison the effect of stilboestrol may last only about 9 days. Quite in contrast to this is the prolonged oestrogenic effect seen after subcutaneous injections of these oestrogens in rats with severed bile ducts. One wonders if this means that subcutaneous absorption is delayed in rats when the bile duct is severed. Although the general condition of these rats, after the bile ducts are severed, is below normal, there is no reason to believe that once the oestrogen is absorbed from the subcutaneous tissues, it would be any different in the blood or tissues from the same oestrogen following intraperitoneal absorption. Also since oestrogens disappear from the blood in a few hours [Rakoff, Cantarow, Paschkis, Hansen & Walkling, 1944] it would seem that delayed absorption offers a tangible explanation for the long duration of oestrus seen in these rats.

Absorption of oestrogens from the bile of the congested bile ducts probably occurs. We have been able to pass stilboestrol (in alkaline solution) and oestrone through the gall bladder of cats in dialysis experiments *in vitro*. In one case a dilated bile duct of an experimental rat was of sufficient size to be used as a dialysis membrane and oestrone dialysed through it. These experiments were conducted in an ice-box at 38° F. for periods of 12–24 hr. It should be mentioned that the bile, taken from the dilated bile ducts of rats, contained oestrogenic activity. Therefore, following injections of stilboestrol and oestrone as was previously mentioned [Emery, 1944], absorption from the bile would be another reason for a more prolonged oestrus in animals with bile ducts severed, but it is difficult to see how this factor would be more effective

after subcutaneous injections. For example, when stilboestrol was given intraperitoneally, oestrus lasted 1.8 days longer in the rats with severed bile ducts but after subcutaneous injection oestrus was 4.6 days longer (Table 1).

#### SUMMARY

The duration of oestrus in rats injected with oestrone or stilboestrol was determined daily by vaginal lavage. The oestrogenic effect of stilboestrol was as usual more prolonged than that obtained with a similar amount of oestrone. When the enterohepatic circulation of oestrogens was eliminated by severing the bile duct the oestrogenic effects of oestrone and stilboestrol were definitely prolonged. Since the result of cutting the bile duct was to prolong oestrus it seems evident that the enterohepatic circulation of stilboestrol does not account for the known long-lasting effect of this oestrogen.

After the bile duct was severed, both oestrone and stilboestrol were especially prolonged in action following subcutaneous injections. This is thought to imply delayed absorption, rather than delayed destruction of these oestrogens by the liver.

We are indebted to Dr Oliver Kamm, Parke, Davis and Co. and to Dr Erwin Schwenk, Schering Corporation for supplies of oestrone and stilboestrol respectively.

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# SOME FACTORS AFFECTING THE ABSORPTION RATE OF SUBCUTANEOUSLY IMPLANTED HORMONE TABLETS

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(Received 21 March 1945)

This and a previous investigation of the kinetics of tablet absorption [Folley, 1944] arose out of the use of the implantation technique of Deanesly & Parkes [1937] for the artificial induction of lactation in cows and heifers by prolonged oestrogen treatment [Folley & Malpress, 1944; Hammond & Day, 1944]. The requisite uptake of synthetic oestrogen (diethylstilboestrol or *meso*-hexoestrol) could be achieved by implanting a few large (1000 mg.) tablets or a considerable number of small (50, 25 or 15 mg.) tablets into the same site or 'pocket', but unabsorbed residues remained to be removed at the end of the treatment period.

It was pointed out by Folley, Stewart & Young [1944] that the whole procedure would be simplified if conditions could be found under which tablets yielding a suitable daily dose of oestrogen would be completely absorbed in the required time, thus obviating the necessity for a second minor operation for their removal. It seemed to them likely that the absorption rate of tablets containing oestrogen diluted with lactose (diluted tablets) would be greater than that of tablets of pure oestrogen, since a highly soluble sugar would be dissolved away soon after implantation [Parkes, 1942], leaving a very porous tablet which should be readily permeable to the solvent body fluids. A further advantage of such tablets lay in the fact that the incorporation into the tablet matrix of a diluent such as lactose and a small quantity of a lubricant such as stearic acid is necessary for the large-scale manufacture of tablets in commercial machines. However, in the event, diluted tablets were found by Folley *et al.* [1944] to be absorbed in bovines even slower than tablets of pure oestrogen.

This at first sight rather anomalous result is explicable on the basis of the phenomenon of 'ghost' formation [Folley, 1942, 1944]. The occlusion even of the relatively large pores of a diluted tablet, from which the lactose had been dissolved away, with highly insoluble protein material would be expected to deny access of the body fluids to the interior of the tablet, thereby restricting absorption to the outer surface. In this connexion, evidence that absorption from compressed tablets of hexoestrol is proportional to surface area, and by inference restricted to the tablet surface, has been presented by Folley [1944]. Thus it might well be that the effective surface area of a diluted tablet would be actually less than that of a compressed tablet of pure hormone\* of the same dimensions, since a greater proportion of the surface of the former would be occupied by inert areas represented by the open ends of pores filled with insoluble protein.

\* In this paper, for convenience, the word hormone is used in respect of both testosterone and hexoestrol, though the latter is not a hormone in the usual sense of the word.

If this theory were correct it might further be expected (1) that cast tablets formed by solidification of fused hormone would exhibit a greater absorption rate per unit area than compressed tablets since cast tablets should contain no pores and hence the whole of the outer surface would be effective as regards absorption, and (2) that the absorption rate per unit area of compressed tablets should, within limits, depend on the compression used in making them, since it would seem likely that highly compressed tablets would be less porous than tablets made with a minimum of compression. The possibility must be borne in mind, however, that the postulated differences in absorption rate exist but are too small to be measured by the techniques available.

This paper records experiments on tablet absorption in rats designed to test both these possibilities together with parallel experiments on the absorption of tablets containing lactose and thereby provides further information on factors governing the absorption of subcutaneously implanted tablets.

#### EXPERIMENTAL

##### *Material and methods*

The experimental animals were hooded Norway rats, males being used in some experiments and females in others. The tablets were implanted subcutaneously in the shoulder region under ether anaesthesia, one into each rat.

Hexoestrol tablets were used for most of the experiments since hexoestrol is relatively cheap and readily available and also because information on the absorption rates of tablets of synthetic oestrogen is of practical importance for both veterinary and medical purposes. In addition, tablets of testosterone were used in some experiments.

All of the cast tablets and many of the compressed tablets were specially made for the work by commercial firms; some of the compressed tablets were made in the laboratory in a special apparatus illustrated in Fig. 1 in which the pressure applied could be accurately controlled. The commercial compressed tablets, with the exception of one batch of testosterone tablets which were true cylinders, had the form of cylinders with convex ends while those made in the laboratory were true cylinders, as were also the cast tablets. Each tablet was weighed and measured at the outset so that the mean surface area for all the tablets used in a given experiment could be calculated, using for the tablets with convex faces, the formula given previously [Folley, 1944]. After removal the tablets were cleaned, dried, weighed and quantitatively extracted with ether as previously described [Folley, 1944]. All absorption data given hereafter are 'true' absorption values [see Folley, 1944] calculated from the weights of the ether extracts. To facilitate comparisons with the data for tablets of pure hormone, absorption data for diluted tablets are given in terms of the weight of hormone absorbed expressed as a percentage of the original weight of the tablet.

##### *Effect of compression on absorption rate*

Five groups of female rats were used for these experiments. The different groups were implanted with series of tablets of pure hexoestrol made in the laboratory under pressures ranging from 12.4 to 62.0 tons/sq. in. (1953–9765 kg./cm.<sup>2</sup>). These limits were imposed by the tendency of tablets prepared at lower pressures to crumble on handling and of the punch to undergo distortion at higher pressures.

The results which are given in Table 1 show no obvious indications of the existence of a correlation between compression and absorption rate, though interpretation is complicated by rather wide divergencies in many cases between duplicate determinations. The mean surface areas for all series of tablets were very nearly equal so that in comparing the various results no correction for differences in area is necessary. Mean values for the tablets prepared under pressures of 12.4, 24.8 and 37.2 tons/sq. in. and 37.2, 49.6 and 62.0 tons/sq. in. respectively are given in Table 1 and plotted in Fig. 2.

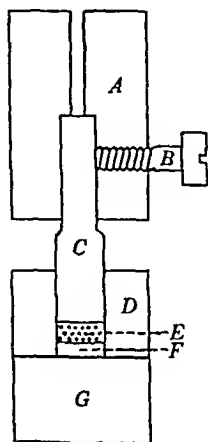


FIG. 1.

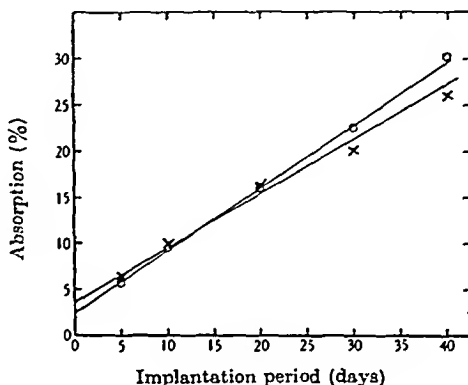


FIG. 2.

FIG. 1. Sectional diagram of apparatus for preparing hormone tablets under uniform compression. The die (D) is placed on the steel block (G) with the bottom punch (F) in position. A weighed amount of the powdered hormone (E) is brushed into the die and the top punch (C), which is held in the punch holder (A) by the set screw (B), inserted. The assembly is then placed in a hydraulic press and the requisite pressure (read by a gauge) applied. To eject the tablet, the block (G) is replaced by a steel block drilled with a hole of diameter greater than that of the punch and just sufficient pressure applied to eject the tablet. With the exception of the punch holder and set screw, which are of mild steel, all components are made of tempered steel.

FIG. 2. Effect of compression on the absorption rate of hexoestrol tablets. The crosses represent the mean values for tablets prepared under pressures of 12.4, 24.8 and 37.2 tons/sq. in. while the circles represent the mean values for tablets prepared under pressures of 37.2, 49.6 and 62.0 tons/sq. in.

Fig. 2 is consistent with the interpretation that the 'high-pressure' tablets have inherently a slightly higher absorption rate than 'low-pressure' tablets but that a labile component [Folley, 1944] is present to a greater extent in the latter, thus giving them a slightly higher initial absorption. In view of the variance of the results, however, it is not justifiable to say more than that they are not inconsistent with the theory put forward in the introduction to this paper. It may well be that the differences in absorption rate expected on the basis of the theory are hardly greater than the biological and other errors inherent in the determinations. One conclusion of practical importance can be drawn however: it is safe to say that, within the range of pressure studied, five-fold variations in compression have no significant effect on absorption rate.

Table 1. *Effect of compression on the absorption rate of cylindrical hexoestrol tablets*

Group	Pressure used in making tablet		No. of tablets	Mean radius mm.	Mean height mm.	Mean surface area mm. <sup>2</sup>	Mean weight mg.	% absorption at day				
	Tons/in. <sup>2</sup>	Total (tons)						5	10	20	30	40
1	12.4	0.74	10	3.50	2.79	138	99.5	5.1	10.0	11.4	12.0	19.2
								5.8	10.7	14.4	21.2	25.7
2	24.8	1.48	10	3.50	2.74	137	103.0	6.6	8.7	17.2	19.4	29.4
								7.0	10.6	17.5	24.4	30.3
3	37.2	2.22	9	3.50	2.71	136	101.6	7.3	9.9	18.1	20.7	26.1
								7.9	9.9	19.4	23.3	—
4	49.6	2.96	10	3.50	2.71	136	102.9	4.6	6.8	10.7	19.8	24.6
								7.2	10.8	18.4	32.6	51.9
5	62.0	3.70	10	3.53	2.70	138	102.5	3.8	10.1	11.9	17.1	19.3
								4.2	10.2	17.7	22.0	29.2
							Mean of (1), (2) and (3)	6.6	10.0	16.3	20.2	26.1
							Mean of (3), (4) and (5)	5.8	9.6	16.0	22.6	30.2

Table 2. *Rates of absorption of testosterone tablets*

Type of tablet	Shape	Mean weight mg.	Mean radius mm.	Mean height mm.	Mean surface area mm. <sup>2</sup>	Initial absorption rate		k
						mg./day	mg./day/mm. <sup>2</sup>	
(1) Cast	Cylindrical	75.2	2.24	4.61	96.1	1.74	0.018	0.0175
(2) Cast	Cylindrical	75.0	1.625	8.325	101.6	2.09	0.021	0.010
(3) Cast*	Cylindrical	100.0	2.50	4.65	112.9	1.11	0.010	0.009
(4) Compressed	Cylinder with convex ends	74.1	2.40	4.14†	86.1	1.74‡	0.020‡	—
(5) Compressed	Cylindrical	74.3	1.63	8.05	99.4	2.09§	0.021§	—

\* Results on men [Bishop & Folley, 1944].  
† This is the height at the centre of the tablet ( $t_2$ ). The mean value of the height at the edge ( $t_1$ ) was 3.23 mm. [see Folley, 1944].  
‡ Estimated graphically (see Fig. 3).  
§ Estimated from similarity of these results to corresponding ones for cast tablets (see Fig. 4).

*Comparison of the absorption rates of cast and compressed tablets*

Tablets of testosterone were used for these experiments because, in the experience of one of us [see Bishop & Folley, 1944], cast tablets of hexoestrol tend to crumble soon after implantation. This may not apply to cast tablets of other synthetic oestrogens or even to small tablets of hexoestrol since Shimkin, Lorenz, Wyman & Norton [1944] successfully used small cast tablets of diethylstilboestrol. All tablets weighed approximately 75 mg. In all, three experiments were performed, in two of which the absorption rate of fused cylinders of which the diameter was approximately equal to the height was compared with that of compressed cylindrical tablets with convex ends of which the diameter was somewhat greater than the greatest height (see Table 2, series 1 and 4 for mean tablet dimensions). The combined results for these two experiments are shown in Fig. 3.

The results with the cast cylinders give a fair fit to the equation

$$A = 100 \left( 1 - \frac{(r-kt)^2 (h-2kt)}{r^2 h} \right),$$

where  $A$  is the percentage absorption at time  $t$  of a cylindrical tablet of initial radius  $r$  and initial height  $h$ , and  $k$  is a constant. This equation, which was deduced by Dr A. C. Bottomley on the assumption that the absorption rate at any instant is proportional to the surface area at that instant, was shown by Bishop & Folley [1944] to fit their data for the absorption of cast cylinders of testosterone implanted into men. The values of the absorption constant  $k$ , which is a measure of the volume of hormone absorbed from unit area in unit time, for the present experiment and as found by Bishop & Folley [1944] are given in Table 2. The absorption rate at any instant is given by

$$\frac{dA}{dt} = \frac{200k (r-kt) (r+h-3kt)}{r^2 h},$$

from which, putting  $t=0$ , the initial absorption rate is given by

$$\frac{200k (r+h)}{r h}.$$

The mean initial absorption rates expressed as mg./day and mg./day/mm.<sup>2</sup> calculated from the values (%/day) obtained by making appropriate substitutions in this formula are also given in Table 2.

At each time interval the mean value for the percentage absorption of the compressed tablets was slightly greater than the corresponding value for cast tablets (Fig. 3). It will also be seen that the absorption curve for the compressed tablets bends upward after some 15–20 days. Apart from this latter complication any attempt to fit an equation to these results meets the difficulty that it is not known whether or not compressed testosterone tablets possess a labile component as do large compressed tablets of hexoestrol [Folley, 1944]. If it be assumed not, which would mean that the absorption curve is a smooth curve passing through the origin, then the initial velocity of absorption of the compressed tablets is clearly a little greater than that of the cast tablets. If, on the other hand, it be assumed that there is a labile component, as these results seem to suggest, and further that as in the case of the hexoestrol tablets the absorption rate during the initial stages is approximately linear,



the initial absorption rate, to a good approximation, is given by the slope of the straight line fitting the initial points. From the slope of the straight line (drawn by eye) fitting the three best defined points, i.e. those which are means of five replicates, the initial velocity of absorption was graphically estimated as 2.35 %/day or 1.74 mg./day, which is identical with the value for the cast tablets (Table 2). Thus in either event, since the mean surface area of the cast tablets, in consequence of their shape, was approximately 11.6 % greater than that of the compressed tablets (Table 2, series 1 and 4), the mean initial rate of absorption per unit area of the latter must have been greater by at least 10 % than that of the former.

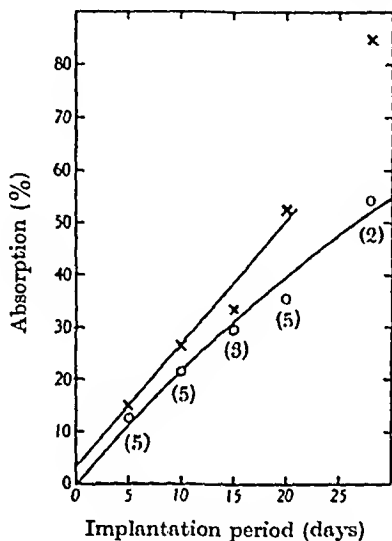


FIG. 3.

FIG. 3. Absorption rates of cast and compressed tablets of testosterone. The circles represent mean values for the percentage absorption from 75 mg. cylindrical cast tablets and the crosses corresponding values for 75 mg. compressed tablets shaped like cylinders with convex ends. The number under each pair of points gives the number of replicate determinations. The equation of the curve fitted to the data for the cast tablets is

$$A = 100 \left( 1 - \frac{(r-kt)^2 (h-2kt)}{r^2 h} \right),$$

where  $A$  = the mean percentage absorption at time  $t$ . The mean initial radius ( $r$ ) of the tablets = 2.24 mm., the mean initial height ( $h$ ) = 4.61 mm., and  $k$  = 0.0175.

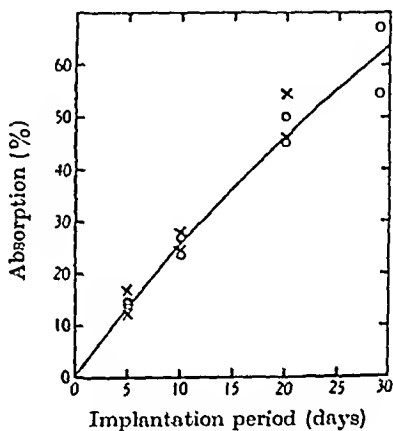


FIG. 4.

FIG. 4. Absorption rates of cast and compressed elongated cylinders of testosterone. The circles represent values for the percentage absorption from 75 mg. cast tablets and the crosses values for 75 mg. compressed tablets. The equation of the curve fitted to the data for the cast tablets is

$$A = 100 \left( 1 - \frac{(r-kt)^2 (h-2kt)}{r^2 h} \right),$$

where  $A$  = the percentage absorption at time  $t$ . The mean initial radius ( $r$ ) of the tablets = 1.625 mm., the mean initial height ( $h$ ) = 8.325 mm., and  $k$  = 0.019.

In the third experiment both types of tablet had the form of elongated cylinders and their mean dimensions were very similar (Table 2, series 2 and 5). The compressed tablets in question were the only set of tablets prepared in commercial machines that were true cylinders. The results are shown in Fig. 4, individual determinations being plotted.

Again the results obtained with the cast tablets were well fitted by the equation given by Bishop & Folley [1944], the value of  $k$  showing fair agreement with the value

obtained for cylinders of different proportions in the previous experiments (Table 2). The values for the compressed cylinders can be reasonably considered as falling on the same curve, indicating that in this experiment the initial velocities of absorption of cast and compressed tablets were approximately equal, though it may be held that the agreement between duplicate determinations was not sufficiently close definitely to exclude the possibility that the true curve might, on extrapolation, make a small positive intercept on the vertical axis.

It will be seen (Table 2) that the values of the absorption constant  $k$  obtained in the present experiments are approximately double the value obtained by Bishop & Folley [1944]. This means that cast cylinders of testosterone are absorbed approximately twice as fast in rats as in men. This fact is also illustrated by the values for the initial absorption rate per unit area given in Table 2.

#### *Absorption of tablets containing lactose*

The results of five experiments in which the absorption rate of tablets of pure hormone was compared with that of tablets containing lactose are given in Table 3. Hexoestrol tablets of various sizes were used in four experiments, and testosterone, as an example of a steroid hormone, in the fifth. The most reliable results are considered to be those of Exp. 3 (Table 3) in which hexoestrol tablets made in the laboratory under uniform compression were used, there being three replicate determinations for each time interval. The individual weights of these tablets were much more uniform than those prepared commercially. In making this batch of tablets particular care was taken to ensure uniform mixing of hexoestrol and lactose. This was tested by selecting six tablets at random and extracting them with water until the extracts gave negative tests for lactose; the tablets were then dried to constant weight in the usual way. The results given in Table 4 show that in all cases the weights of the extracted tablets were a little less than 50% of the original tablet weights. These results may be considered satisfactory; the small discrepancy can be accounted for by the loss of a small quantity of hexoestrol (labile component) when hexoestrol tablets are soaked in water [Folley, 1944].

The tablets used in the rest of these experiments were prepared in commercial machines. The diluted hexoestrol tablets which are given in Table 3 as containing 49% lactose, also contained 1% stearic acid, added to the mixture as a lubricant, in addition to 50% hexoestrol. In one of these experiments (Exp. 4, Table 3) the experimental plan necessitated the selection at random of a number of diluted tablets from a batch and the aqueous extraction of the lactose before implantation. The mean initial weight of these extracted tablets was very near the theoretical value of 51% of the mean weight of a similar number of untreated tablets from the same batch (Table 3) which indicates that the substances used in the preparation of this batch of commercial tablets were uniformly mixed.

The results shown in Table 3, taken as a whole, show no evidence that tablets containing 25, 49 or 50% lactose are absorbed more rapidly than tablets of pure hormone even when, in the case of Exp. 4, allowance is made for the fact that the mean surface area of the tablets of pure hexoestrol was about 15% greater than the values for the diluted tablets. Similar results were obtained with diluted tablets from which the lactose was extracted before implantation (Exp. 4). Indeed in all the experiments

Table 3. *Comparison of absorption rates of tablets of pure hormone and tablets containing hormone diluted with lactose*

Substance	Total no. of tablets	Mean initial wt. of tablets mg.	Mean surface area mm. <sup>2</sup>	Lactose content of tablets %	Mean % absorption* at day								Mean wt. of ghosts mg.
					3	5	10	20	28	30	40	41	
(1) Hexoestrol	10	44.0	79.6	0	—	5.9	13.2	23.7	—	28.4	38.8	—	—
	10	46.1	76.0	49	—	5.1	10.3	14.3	—	27.7	32.3	—	—
(2) Hexoestrol	12	106.6	134.9	0	1.1	2.5	4.5	7.9	—	14.9	—	14.5	0.4
	12	107.0	133.4	25	4.8	4.3	6.1	8.4	—	13.3	—	15.8	2.7
(3) Hoxoestrol	12†	100.2	130.5	0	—	3.3	7.7	17.8	—	19.4	—	—	0.8
	12‡	101.2	123.8	50	—	5.0	7.2	14.2	—	18.5	—	—	1.4
(4) Hexoestrol	12	204.8	232.8	0	2.6	2.9	5.5	12.0	—	13.2	10.7	—	2.9
	12	237.9	207.9	49	4.2	4.3	6.1	8.5	—	14.5	16.8	—	6.8
	10‡	238.5§	207.9	49	2.2	2.7	3.1	8.1	—	—	14.0	—	6.1
(5) Testosterone	8	74.3	85.3	0	—	14.3	25.1	62.4	84.8	—	—	—	—
	8	73.9	82.5	25	—	12.4	25.3	45.8	64.7	—	—	—	—
	8	74.2	81.0	50	—	12.3	19.0	42.8	38.6	—	—	—	—

\* In the case of tablets containing lactose the figures given represent the absorption of hormone expressed as a percentage of the initial weight of the tablet.

† Tablets made in the laboratory under a pressure of 24.8 tons/in.<sup>2</sup>

‡ Tablets from which the lactose was extracted with water before implantation. The figures given represent the absorption of hexoestrol expressed as a percentage of the initial weight of the tablet before water extraction.

§ Twice the mean initial weight of the water-extracted tablets.

Table 4. *Dry weights of hexoestrol tablets originally containing 50 % lactose, after extraction with water*

Tablet no.	...	...	...	1	2	3	4	5	6
Initial wt. (mg.)				103.5	103.2	102.3	102.3	102.0	102.6
Wt. after water extraction (mg.)				50.0	49.9	49.7	50.2	50.3	50.1

with hexoestrol there appears to be no significant difference between the absorption rates of the two types of tablet except that some of the results indicate that diluted tablets are absorbed slightly faster in the very early stages. It seems likely that ghost formation is not complete for some days after implantation, so that for a short time the diluted tablets would be relatively more porous than the tablets of pure hormone.

On the other hand, the results obtained with testosterone, which is absorbed much more rapidly than hexoestrol, so that absorption had progressed much further during the experimental period, indicate that, save for the initial stages, the absorption of hormone from diluted tablets was slower than from tablets of pure hormone, the difference becoming more marked as absorption progressed.

Some values for the mean weights of the ghosts are given in Table 3. As absorption proceeds and the tablets become smaller the ghosts must also progressively decrease in size, so that the mean value of the weights of the ghosts present in all of the tablets used in a given experiment has little significance. In order to provide a sound basis for comparison only values from tablets which had undergone no more than 10% absorption were used and only experiments yielding at least six such values included. It will be seen that in agreement with the findings of Folley *et al.* [1944], and as might be expected, the ghosts present in diluted tablets were heavier than those formed in tablets of pure hormone.

#### DISCUSSION

The present experiments have shown that considerable variations in the pressure used in making compressed tablets of hexoestrol have little effect on the subcutaneous absorption rate of the tablets. Consideration of the mean tablet dimensions given in Table 1 leads to the conclusion that hexoestrol is not very compressible over the range of pressures studied and it seems likely that there was little difference in mean pore size between tablets prepared under the highest and lowest pressures used. This would imply that if the theory proposed in the introduction to this paper were correct, the expected differences in absorption rate might well be too small to detect with the methods available. The analysis of the results as presented in Fig. 2 does suggest, however, that there may be a slight decrease in the absorption rate per unit area as compression is increased, but in view of the magnitude of the experimental errors involved this conclusion can only be accepted with reserve. The fact that the influence of compression on absorption rate is at the most small, and of no significance in practice, means that there is no need for the use of elaborate apparatus to ensure uniform compression such as that described by Forbes [1941].

It was also found that there is no very great difference between the absorption rates per unit area of cast and compressed tablets of testosterone. This result is in general agreement with the findings of Deanesly & Parkes [1943] with tablets of oestradiol, testosterone, progesterone and deoxycorticosterone acetate and of Shimkin & Zon [1943] with diethylstilboestrol tablets. Here again it must be emphasized that our results do not exclude the possibility of the existence of differences in absorption rate,

such as postulated by the theory under discussion, so small that they need a more refined technique for their detection.

The present data provide confirmation of the mathematical law governing the absorption of cast cylinders of pure testosterone propounded by Bishop & Folley [1944]. This law, which was deduced on the assumption that the absorption rate at any instant is proportional to the surface area at that instant, was confirmed for cast cylinders of diethylstilboestrol by Shimkin *et al.* [1944]. It follows that encapsulation of the tablet, which probably always occurs, has no effect on absorption rate, the observed falling off in the latter being solely due to the decrease in surface area. It seems likely that cast tablets will come into increasingly wide use for clinical purposes, since not only are they easily prepared by a procedure which automatically involves heat sterilization but they are absorbed relatively uniformly at a progressively decreasing rate which is predictable, so that, as pointed out by Shimkin *et al.* [1944], a given daily uptake can be maintained within fairly narrow limits by supplementary implants.

The present results indicate a value for the initial velocity of absorption of cast cylinders of testosterone in rats which is approximately twice the value found for men by Bishop & Folley [1944], a finding of considerable interest since it suggests the possible existence of a correlation between absorption rate and the size, or perhaps basal metabolic rate, of the test animal. No indication of such a correlation had, however, been found by Folley [1944] who studied the absorption of compressed hexoestrol tablets in a variety of species ranging in size from rats to cows. This question is of obvious importance and needs further investigation since clinical treatment by tablet implantation is often based on absorption data obtained on rats.

Since we have found the absorption rate of compressed tablets to be approximately the same as that of cast tablets of comparable area, at any rate during the initial stages, it follows that the absorption of the former must be largely restricted to the surface and it may therefore be inferred that ghost formation, which occurs in compressed but not cast tablets [Deanesly & Parkes, 1943; Bishop & Folley, 1944], does affect the absorption of the former by occluding the pores and thus preventing absorption from the interior of the tablet.

This conclusion is supported by the results of the experiments on the absorption of tablets containing lactose. In most of the experiments described above, the latter were absorbed at approximately the same rate as control tablets of pure hormone. In no case were diluted tablets absorbed faster than tablets of pure hormone as would be expected if ghost formation did not occur. Since tablets containing 50% lactose contain only half as much hormone as undiluted tablets of equal weight, it follows that if the absorption rates of the two types of tablet (in both cases calculated in terms of the weight of hormone absorbed daily expressed as a percentage of the original tablet weight) remain equal throughout, tablets containing 50% lactose will be completely absorbed in about half the time necessary for the complete absorption of undiluted tablets. This state of affairs does not appear to be realized in practice however, since in the experiments with diluted testosterone tablets the absorption rate of diluted tablets, at first not very different from that of tablets of pure testosterone, in the later stages became appreciably less than that of the latter in agreement with the theory put forward earlier in this paper. In this respect, these results are in harmony with those of Folley *et al.* [1944] with diluted tablets of hexoestrol implanted into

bovines for periods which, and this may be significant, for the most part exceeded any in the present experiments.

It may be said that the present results, taken as a whole, while not agreeing in all details with the above-mentioned theory, probably because the differences sought are too small to be detected by the experimental techniques used, are in general harmony with the conception that ghost formation exerts an influence on the absorption rate of compressed hormone tablets, made up with or without the addition of lactose, by restricting absorption to the tablet surface.

#### SUMMARY

1. The influence of various factors on the absorption rates of tablets of hexoestrol and testosterone in rats has been studied.
2. Over a pressure range of 12.4–62.0 tons/sq. in. the absorption rate of compressed hexoestrol tablets was for all practical purposes independent of the pressure used in making them.
3. The initial absorption rates per unit area of cast and compressed tablets of testosterone were approximately equal.
4. The absorption rate of cast cylinders of testosterone obeys a mathematical law deduced on the assumption that absorption rate at any instant is proportional to the surface area at that instant.
5. The value found for the initial absorption rate of cast tablets of testosterone in rats was approximately twice the value found previously in men.
6. Tablets of hexoestrol and testosterone containing 25, 49 or 50 % lactose (diluted tablets) were absorbed at approximately the same rate as, certainly no faster than, tablets of pure hormone. During the later stages of absorption, the absorption rate of diluted tablets of testosterone was much less than that of tablets of pure hormone.
7. The probable effects of ghost formation on the absorption of compressed tablets are discussed in the light of the above findings.

We are indebted to Dr S. K. Kon for placing the facilities of the rat colony maintained by him at our disposal; to Messrs Organon Laboratories, Ltd., for the gift of numerous testosterone tablets made to our specification and to Messrs Schering Ltd. for one batch of compressed testosterone tablets supplied before the war. Our thanks are also due to Mr S. C. Watson for skilled technical assistance and in particular for making the special apparatus for preparing tablets under uniform compression. This work was carried out during the tenure by A.T.C. of a Research Grant from the Agricultural Research Council.

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## SOME FACTORS AFFECTING ABSORPTION FROM IMPLANTED TABLETS

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(Received 21 March 1945)

Folley & Malpress [1944] and Hammond & Day [1944] have recently described the use of the tablet implantation technique for the prolonged administration of oestrogens to cattle. This work, which was carried out in connexion with extensive experiments on the induction of lactation in sterile heifers, raised certain new problems relative to the technique. Chief among these problems were the following.

(a) The large-scale production of the tablets one by one in a hand-operated tablet-making machine proved impossibly laborious. The use of a commercial mechanically operated machine, however, requires that the powder to be tableted shall have certain physical properties, which are usually obtained by the addition of a diluent such as lactose, and a process of granulation. The necessary properties are not possessed by the undiluted microcrystalline oestrogen powders, and the commercial machines are used only with difficulty for such material. The use of tablets containing lactose as a diluent and stearic acid as a lubricant was therefore investigated. These tablets did not prove very satisfactory in cows [Folley, Stewart & Young, 1944].

(b) The dose required by the cows was large, of the order of 2.5 g., and necessitated the implantation of one or two very large tablets, the behaviour of which was at that time comparatively unexplored, or of a large number of small ones. It was obviously inconvenient to make more than one incision for implantation, but it was found that a large number of small tablets (e.g.  $50 \times 50$  mg. or  $160 \times 15$  mg.) put into one subcutaneous pocket tended to mat together, a fact that appeared to explain their comparatively slow absorption. In rats, a 15 mg. tablet of diethylstilboestrol individually implanted is mostly absorbed in one month. In cows the mass of residual tablets, even of this small size, usually had to be removed surgically when it was desired to discontinue treatment at the end of the required period, 60 or 90 days.

(c) The minor surgery involved by the implantation and subsequent removal of the tablets was inconvenient and it was thought that intraperitoneal implantation through a trocar might be more simple and might facilitate absorption of the whole of the tablet in the required time.

It seemed that these problems, the effect of dilution and of crowding on absorption rate, and the potentialities of intraperitoneal implantation, would be susceptible to investigation in laboratory animals. The following paper records results obtained with tablets of diethylstilboestrol and hexoestrol, with or without diluent, implanted subcutaneously or intraperitoneally into rats. At the same time some other experiments were done to find out if intraperitoneal implantation would accelerate the absorption of certain substances which are taken up very slowly when implanted subcutaneously [Parkes, 1942].

## MATERIAL AND TECHNIQUE

Adult male rats were used. Implantations were made under ether anaesthesia. For subcutaneous implantations a skin incision was made, usually in the flank, and a pocket hollowed out in the subcutaneous tissue for the reception of the tablets. For intraperitoneal implantations incisions were made in both the skin and the body wall and the tablets were dropped into the peritoneal cavity. All incisions were finally closed with sutures.

*Substances used*

Tablets of the following composition were used.

Hexoestrol	%	Diethylstilboestrol	%
Hexoestrol	100	Lactose	50
Lactose	60	Stearic acid	49
Stearic acid	39		1
Hexoestrol	1	Diethylstilboestrol dipropionate	60
Lactose	50	Lactose	39
Stearic acid	49	Stearic acid	1
Diethylstilboestrol	1	Dehydro- <i>iso</i> -androsterone	100
Diethylstilboestrol	100	Androstenedione	100
Lactose	60	Pregneninolone	100
Stearic acid	39	Thyroxine	100
	1		

*Estimation of absorption*

After removal the tablets were dissected free from connective tissue, lightly washed, dried and weighed. Absorption was calculated by expressing, to the nearest whole number, the loss of active substance as a percentage of the amount originally present. It has been shown by Folley [1942] that tablets thus recovered contain an interstitial deposit of protein matter (the ghost) which can be demonstrated by dissolving the active material in ether or other appropriate solvent, and which slightly complicates the calculation of absorption rate. When the interstitial spaces are relatively large owing to the removal of the readily absorbed lactose the protein deposit even in a small tablet (50 mg. original weight) may weigh 2 mg. [Deanesly & Parkes, 1943]. It was thought unnecessary to obtain the weight of this ghost in every case, since, in general, the conclusions reached are not dependent on small differences.

## EFFECT OF DILUENT

Previous work [Parkes, 1942] showed that a highly soluble substance such as lactose is rapidly extracted from a tablet implanted subcutaneously, even in the presence of an excess of a highly insoluble constituent such as cholesterol. It was not surprising, therefore, to find, in an initial series of experiments (Table 1), that the tablets containing hexoestrol or diethylstilboestrol and lactose lost, within 5 days, an amount closely corresponding with the original lactose content. The residue of the tablet at this stage would consist of any unabsorbed lactose, a small amount of stearic acid, which is not significantly absorbed from the subcutaneous tissues, almost all the original content of active substance of which little will have been absorbed at that stage, and a 'ghost', deposition of which will already have started. In practice it has been impracticable to allow for minor variables, and throughout the experiments the



weight of the tablet after 5 days' or longer implantation has been taken as residual diethylstilboestrol. After the absorption of the lactose, especially where it was originally present to the extent of 50 %, the tablet contains extensive interstices, and is a highly porous body. It is, nevertheless, firm and coherent. It seemed likely that the structural condition would facilitate absorption.

Table 1 shows that, on subcutaneous implantation, the rate of absorption of the active substance in the initial experiments did not exceed 25 % in 20 days from tablets containing 49 % of lactose. This rate was exceeded in one of the later experiments recorded in Table 3, but comparison with the data for undiluted diethylstilboestrol or hexoestrol, also given in Table 3, suggests that the porous tablets of active substance left after the removal of the lactose are not absorbed more rapidly than tablets of the pure substance, and may be absorbed more slowly. Implantations of 40 days' duration, as well as reimplantations of tablets removed after 10 days, lead to a similar conclusion.

Table 1. *Absorption from tablets containing diluent*

Nature of tablet	No. of tablets	Days implanted	Wt. of tablet (mg.)		Active substance absorbed %
			Initial	Final	
Hexoestrol 50 %	5	5	50	24	4
Lactose 49 %	5	10	50	22	12
Stearic acid 1 %	5	20	50	19	24
	2	5	250	124	1
	2	24	250	105	16
	2	31	250	49	61
Hexoestrol 60 %	5	5	50	30	0
Lactose 39 %	5	10	50	28	6
Stearic acid 1 %	5	20	50	26	13
Diethylstilboestrol 50 %	5	5	50	25	0
Lactose 49 %	5	10	50	23	8
Stearic acid 1 %	5	20	50	21	16
Diethylstilboestrol 60 %	5	5	50	31	0
Lactose 39 %	5	10	50	29	3
Stearic acid 1 %	5	20	50	29	3
Diethylstilboestrol dipropionate 60 %	5	5	50	30	0
Lactose 39 %	5	10	50	29	3
Stearic acid 1 %	5	20	50	26	13

This result accords with those obtained in cows by Folley, Stewart & Young [1944] who found that absorption was slower from tablets containing 50 % hexoestrol than from those containing 100 %. Probably the replacement of the lactose by the protein deposit, which is highly insoluble [Folley, 1944], serves to inhibit to a lesser or greater extent the absorption of the diethylstilboestrol or hexoestrol. The idea of such an inhibitory action is quite compatible with the fact that the weight of the interstitial protein deposit, as determined in the dried residue, is relatively insignificant. The 'ghost' is more substantial in the porous residues of the tablets originally containing diluent than in the less porous residues of tablets composed only of oestrogen [Deanesly & Parkes, 1943], and it may be inferred that the inhibition of absorption is consequently greater.

In general, the tablets containing 60% of active substance were absorbed less rapidly than those containing 50 or 100%. This result is curious and suggests that some special complication is involved in the absorption of tablets containing diluent.

## CROWDING OF TABLETS

A pocket which can easily be made in the subcutaneous tissues of the rat holds 5 x 50 mg. tablets fairly easily, but the tablets are inevitably crowded together closely. The same applies, though less strongly, to smaller tablets. The effect of this crowding on absorption was investigated by comparing the loss of weight over periods up to 20 days from tablets implanted individually with that from tablets crowded together five per pocket. The results are shown in Table 2, from which it will be seen that in all five

Table 2. *Effect on absorption of number of tablets per pocket (subcutaneous)*

Nature of tablet	Days implanted	No. of tablets	Initial wt. (mg.)	Final weight (mg.)		Active substance absorbed (%)	
				One per pocket	Five per pocket	One per pocket	Five per pocket
Diethylstilboestrol 100%	10	5	25	20	21	20	16
	20	5	25	14	17	44	32
	20	5	50	42	—	16	—
	20*	5	50	38	—	24	—
Hexoestrol 100%	5	5	15	12	13	20	13
	10	5	15	11	12	27	20
	20	5	15	9	11	40	27

\* Five tablets implanted in separate pockets in one rat.

experiments absorption was greater from the individually implanted tablets. Moreover, for both diethylstilboestrol and hexoestrol, the difference was greater at 20 days. Most of the experiments on individual implantation were carried out by implanting one tablet per rat, but the experiment in which five tablets were implanted individually into five separate pockets in the same animal gave similar results, and it is unlikely that the decreased absorption associated with crowding was due to saturation of the body fluids of the host, a possibility suggested by Folley [1944] to explain the comparatively slight absorption from very large tablets in rats.

## INTRAPERITONEAL IMPLANTATION

Absorption from the peritoneal cavity is generally more rapid than from the subcutaneous spaces, and in addition the movements of the viscera might well assist absorption of the tablets. It was expected, therefore, that tablets implanted intraperitoneally would be absorbed more rapidly. However, the ultimate location of such tablets varied considerably. Many were found loose in the abdominal cavity even after a month or more, some were caught up in the omentum fat, and a few were attached to the perinephric fat or even to the liver, which seemed in such cases to have undergone modification. No doubt this variability in location was to some extent responsible for the considerable variation observed in absorption rates.

The full comparison of subcutaneous and intraperitoneal rates of absorption is given in Table 3. It will be seen that on the basis of percentage loss of weight of active

Table 3. *Comparison of subcutaneous and intraperitoneal implantation*

Nature of tablet	Days implanted	Total number of tablets		No. of tablets		Initial wt. (mg.)	Average final weight (mg.)		Average % active substance absorbed	
		s.c.*	I.P.*	per rat	per pocket s.c.*		s.c.*	I.P.*	s.c.*	I.P.*
Exp. 1: Diethylstilboestrol 100 %	5	5	4	1	1	25	22	21	12	16
	10	5	4	1	1	25	20	18	20	28
	20	5	5	1	1	25	14	15	44	40
Exp. 2: Diethylstilboestrol 100 %	15	5	5	5	5	50	41	35	18	30
	20	5	5	5	5	50	42	33	16	34
Exp. 3: Diethylstilboestrol 50 % Lactose 49 % Stearic acid 1 %	15	5	5	5	5	50	20	19	20	24
	20	5	5	5	5	50	15	15	40	40
Exp. 4: Diethylstilboestrol 80 % Lactose 39 % Stearic acid 1 %	10	5	5	5	5	50	29	22	3	27
	20	5	5	5	5	50	28	19	7	37
	40	5	5	5	5	50	15	10	50	67
Exp. 5: Hexoestrol 100 %	5	5	5	1	1	15	12	12	20	20
	10	5	4	1	1	15	11	10	27	33
	20	5	5	1	1	15	9	9	40	40
Exp. 6: Hexoestrol 100 %	5	5	5	5	5	15	13	12	13	20
	10	10	4	5	5	15	11	11	27	27
	20	—	5	5	—	15	—	8	—	47
Exp. 7: Hexoestrol 50 % Lactose 49 % Stearic acid 1 %	10	5	5	5	5	50	22	19	12	24
	20	5	5	5	5	50	21	12	16	52
	40	5	4	5	5	50	20	6	20	76
Exp. 8: Hexoestrol 60 % Lactose 39 % Stearic acid 1 %	10	5	5	5	5	50	28	24	7	20
	20	5	5	5	5	50	23	19	23	37
	40	5	5	5	5	50	19	6	37	80
Exp. 9: Dehydro- <i>iso</i> -androsterone	20	3	4	3	3	50	38	36	24	28
Exp. 10: Androstenedione	20	3	5	3	3	50	39	29	22	42
Exp. 11: Thyroxine	30	5	5	1	1	50	49	49	2	2

\* s.c. = subcutaneous; I.P. = intraperitoneal.

substance from the tablet the intraperitoneal rate was always as great as the subcutaneous rate, and was often considerably greater. In three of the four experiments with tablets containing synthetic oestrogen without diluent there appeared to be little difference in absorption rate. These three included both experiments in which only one tablet per rat was implanted and in which, therefore, subcutaneous absorption would not be retarded by crowding. It may be assumed that the crowding effect would not be found with five tablets scattered in the intraperitoneal cavity. In one experiment in which five tablets were implanted together subcutaneously and intraperitoneally, the intraperitoneal rate was much greater. In three of the four experiments with tablets of oestrogen and diluent, intraperitoneal absorption was also much greater, though in Exp. 7 the excessive difference was probably partly due to the unusually low rate of subcutaneous absorption.

Experiments with other substances gave similar results. Intraperitoneal absorption was considerably greater in one of the two experiments with androgens. The absorption of thyroxine, which is taken up very slowly from tablets, was not increased to any useful extent by intraperitoneal administration. Another experiment, not listed in the table, showed that the absorption of pregnenolone in tablet form from the subcutaneous space, which in our experience is insignificant, is not materially increased by intraperitoneal administration.

#### SUMMARY

1. A study has been made of various modifications of the technique of administering biologically active substances by the implantation of tablets. Most of the experiments were carried out with tablets containing hexoestrol or diethylstilboestrol alone, and with tablets containing hexoestrol or diethylstilboestrol together with 39 or 49 % lactose as diluent and 1 % stearic acid as lubricant.
2. Absorption of the active substance from diluted tablets implanted subcutaneously into rats was no more rapid, and was probably less rapid, than from tablets not so compounded.
3. Absorption was retarded by the crowding together of several tablets into one subcutaneous pocket.
4. Absorption is at least as rapid from intraperitoneally as from subcutaneously implanted tablets, and may be much more rapid.

The intraperitoneal experiments were undertaken at the suggestion of Mr J. Hammond, Jr.

Acknowledgements are due to Boots Pure Drug Company, who prepared the tablets containing diluent and lubricant.

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# FUNCTIONAL RELATION BETWEEN THE UTERUS AND THE CORPUS LUTEUM

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(Received 19 April 1945)

Since Loeb [1923] found that hysterectomy in the guinea-pig causes a marked prolongation of the life of the corpus luteum, the relation between the uterus and the corpus luteum has been the subject of study by many investigators. Loeb [1923, 1927] explains his results by assuming that the uterine endometrium may secrete a certain hormone-like substance which shortens the life of the corpus luteum. Loeb's observation was confirmed by Asdell & Hammond [1933] in the rabbit. Sessums & Murphy [1933] reported that hysterectomy in the rabbit inhibits the development of oestrus and causes degenerative changes of the ovary. Transplantation of the uterine endometrium tends to check the inhibitory and degenerative changes. They thought that the uterine endometrium might elaborate a hormone which influences ovarian activity. In the ferret, however, Deanesly & Parkes [1933] failed to demonstrate any significant influence of hysterectomy on ovarian function. The same operation did not produce luteal persistence in the oestrous rats, but it did so in the pregnant ones [Hechter, Fraenkel, Lev & Soskin, 1940].

In the pregnant rabbit, hysterectomy during the first half of the gestational period shortens the life of the corpus luteum; operation performed at a late stage of pregnancy initiates a precipitous decrease in size of the corpora lutea [Greep, 1941]. This clearly indicates that the uterus-corpora lutea relation may become much more complicated during gestation. The question whether or not the corpora lutea in both pregnant and pseudopregnant conditions are regulated by the same mechanism still requires detailed study.

## MATERIAL AND METHODS

Rabbits were used for the experiments. Animals obtained from a local dealer were usually kept in the laboratory for at least one week before being subjected to experiment. Pseudopregnant females were rendered available by an infertile mating with a vasectomized male. Hysterectomy was performed aseptically at various intervals after coitus.

The experimental animals were divided into seven groups. (1) Pseudopregnant rabbits were hysterectomized at various intervals after mating. (2) Hysterectomized-pseudopregnant rabbits were given auto-transplants of the uterus. (3) Intact pseudopregnant rabbits were given injections of oestrogen. (4) Pregnant rabbits were hysterectomized at various stages of gestation. (5) Transplants of placenta were made in hysterectomized-pregnant animals. (6) Extirpation of the placenta was performed in pregnant rabbits leaving the uterus intact. (7) Pregnant animals were injected with oestrogen after they had been hysterectomized. The survival periods of the corpora lutea in all the experimental animals were carefully determined by laparotomy.

The criteria used for the judgement of complete regression of the corpus luteum were its total deprivation of vascularization and/or its disappearance as a discrete body. Since the changes of the corpora lutea under various conditions of experimentation were so marked, it was unnecessary to section the ovary to determine the exact time of luteal involution. Other details of the experiments will be described under separate headings.

## RESULTS

*Effect of hysterectomy on the corpora lutea of pseudopregnant rabbits*

The uteri of five animals were removed at intervals from 11 to 14 days after coitus. Subsequent laparotomies revealed that the corpora lutea in these animals survived for an average of 15 days (11–18) after hysterectomy. The average total life span was 27.2 days (28–29) (Table 1). It is well known that the average functional period of the corpora lutea in pseudopregnant rabbits is about 16 days. It was therefore lengthened by about 11 days in the hysterectomized animals. Two of these animals were mated again and ovulated. Their corpora lutea lasted 40 and 51 days respectively.

All the experimental animals came into oestrus at various times after hysterectomy and mated when a buck was introduced. Their ovaries contained many follicles of different sizes; they did not show any degenerative signs. Saline extracts had been prepared from their pituitaries and tested on oestrous rabbits. They caused a high percentage of ovulation in the test animals. The conclusion reached by Sessums & Murphy [1933] is obviously at variance with the results of the present study.

Table 1. *The survival period of the corpora lutea in hysterectomized-pseudopregnant rabbits*

Rabbit no.	Time of hysterectomy after coitus days	Survival of corpora lutea after operation days	Total life of corpora lutea days	Deviation of life of corpora lutea from norm days
R15	12	15	27	+ 11
R28	11	18	29	+ 13
R61	14	11	25	+ 9
R10	10	17	27	+ 11
R38	14	14	28	+ 12
Average		15	27.2	

*Effect of uterine implants on the corpora lutea in hysterectomized-pseudopregnant rabbits*

If the uterus conditions the functional period of the corpus luteum, an alternative procedure, i.e. the implantation of the uterus into a hysterectomized-pseudopregnant rabbit, should reduce the survival period of the corpus luteum. This was found to be the case.

Six pseudopregnant rabbits were hysterectomized at various intervals after coitus. The uterine horns thus removed were turned inside out through a longitudinal incision and implanted into the abdominal cavity. The uterine endometrium was thus in direct contact with the visceral organs. The life span of the corpora lutea in these animals, as subsequently determined, showed considerable variation, but in all cases except one, the survival of the corpora lutea appeared to be within the range

of normal variation (Table 2). It may be concluded that auto-transplantation of the uterus in a hysterectomized-pseudopregnant rabbit shortens the life of the corpora lutea approximately to that of normal pseudopregnancy.

Table 2. *The survival period of the corpora lutea in hysterectomized-pseudopregnant rabbits with uterine implants*

Rabbit no.	Time of hysterectomy after coitus days	Survival of corpora lutea after operation days	Total life of corpora lutea days
R 91	10	11	21
R 74	9	7	16
R 94	10	7	17
R 95	9	9	18
R 99	8	17	25
R 53	12	10	22
Average		10	19.8

*Effect of oestrone injection on the corpora lutea of pseudopregnant rabbits*

It has been shown that oestrogen is able to stimulate and sustain the corpus luteum in hypophysectomized rabbits [Robson, 1937]. If the regression of the corpora lutea in pseudopregnant animals is due to a gradual decrease in oestrogen concentration, then an increase should prevent the regression.

Six pseudopregnant rabbits were injected with different doses of oestrone at various intervals after coitus. It was found that a dose of 0.5 mg. of oestrone daily was not adequate to maintain the corpora lutea for the expected length of time; with 1 mg. daily, the corpora lutea could be adequately maintained as long as the injections lasted, and a complete regression of the corpora lutea occurred 3 or 4 days after stopping the injections (Table 3). It is evident that the life span of the corpora lutea lengthens as the oestrogen content in the blood is raised.

Table 3. *The survival period of the corpora lutea in oestrone-injected pseudopregnant rabbits*

Rabbit no.	Time of treatment after coitus days	Dosage of oestrone mg./day	Injection period days	Life of corpora lutea days	Regression of corpora lutea after injection days
R 64	13	1	8	28	7
R 63	10	1	9	—	Disappeared on 9th day of injection
R 72	10	0.5	9	20	Became very small at the end of injection
R 74	10	0.5	9	—	Disappeared at the end of injection
R 78	14	1	10	27	3
R 98	14	1	9	26	3

*Effect of hysterectomy on the corpora lutea of pregnant rabbits*

The results of the foregoing experiments indicate that the function of the corpus luteum is conditioned by the uterus: the presence of uterine tissue shortens the life of the corpus luteum. Two alternative explanations may be suggested. Either the uterine endometrium may elaborate some hormone-like substance which inhibits luteal formation or the uterus may share a common stimulating substance with the corpus luteum, and the removal of the former would render that part of the substance available for the latter, thus lengthening its survival. However, neither of these

two assumptions would explain satisfactorily the behaviour of the corpus luteum during gestation. According to Loeb [1923], the uterus of pregnancy is no longer capable of producing luteal inhibitory substance as a result of the growth of the foetuses, and thus a condition is created which is similar to that after hysterectomy. Loeb's hypothesis appears untenable in view of the findings of Greep [1941], who showed that the removal of the uterus in pregnant rabbits shortens the life of the corpus luteum. It is therefore interesting to ascertain whether the functional maintenance of the corpus luteum in both pregnant and pseudopregnant states is effected by a common mechanism.

Six oestrous rabbits were rendered pregnant and hysterectomized 7-14 days after coitus. The results of extirpation of the uterus appeared somewhat variable, but there was a general tendency towards shortening the functional period of the corpus luteum. The average duration of this period under this condition was 20.5 days (16-26), the average survival period after hysterectomy being 9.5 days (6-12). No corpora survived as long as those of pregnancy, which survive about 28 days (Table 4). It is evident that the removal of the uterus together with the uterine contents in pregnant animals causes a shortening of the functional life of the corpora lutea. The results reported by Greep [1941] are therefore confirmed.

Table 4. *The survival period of the corpora lutea in hysterectomized-pregnant rabbits*

Rabbit no.	Time of hysterectomy after coitus days	Survival of corpora lutea after operation days	Total life of corpora lutea days	Deviation of life of corpora lutea from norm days
R6	14	7	21	- 7
R18	14	12	26	- 2
R23	7	12	19	- 9
R65	7	9	16	-12
R192	12	6	18	-10
R193	12	11	23	- 5
Average		9.5	20.5	

*Effect of placental implants on the corpora lutea of the hysterectomized-pregnant rabbits*

That removal of the uterus shortens the functional period of the corpora in pregnant animals suggests that the mechanism of functional regulation of the corpus luteum in pregnant animals is different from that in pseudopregnancy. Since the removal of the uterus together with the uterine contents definitely shortens, instead of lengthens, the life of the corpora lutea, the factor responsible for the maintenance of this organ must be present in some tissue within the uterus. It is probable that the placenta may be the organ concerned.

Eight pregnant rabbits were hysterectomized at about the mid-term of gestation, and the placenta in each case was removed and implanted into the abdominal cavity at the lumbar level. In a few cases, additional pieces of placental tissue were implanted under the skin of the abdomen or in the region of the mammary glands. The corpora lutea were frequently examined by laparotomies. It was found that in all the cases the life of the corpora was much lengthened, being maintained as long as in normal pregnancy. The average life of the corpora was 28.7 days (25-34), and



the average survival after operation was 14.1 days (12-19) (Table 5). The results furnish evidence that the placenta may provide a stimulus for the maintenance of the corpus luteum.

Table 5. *The survival period of the corpora lutea in hysterectomized-pregnant rabbits with placental implants*

Rabbit no.	Time of hysterectomy after coitus days	Site of placental implantation	Survival of corpora lutea after operation days	Total life of corpora lutea days
R 85	14	Abdominal	12	26
R 88	15	"	17	32
R 89	15	"	14	29
R 90	17	"	11	28
R 28	15	"	19	34
R 215	13	Abdominal and subcutaneous	12	25
R 216	14	Abdominal and mammary region.	15	29
R 220	14	"	13	27
Average			14.1	28.7

*Effect of removal of placenta on the corpora lutea of pregnant rabbits*

Since the removal of the uterus and uterine contents in the pregnant rabbits shortens, while the implantation of placenta in the hysterectomized-pregnant animals lengthens, the survival of the corpora lutea, it is quite evident that the placenta plays an important role in the functional regulation of the corpus luteum during gestation. But as the results of hysterectomy experiments might be complicated by the simultaneous removal of both uterus and placenta, it is advisable to remove the placenta alone and leave the uterus intact in order to obtain a more clear-cut picture.

Six pregnant rabbits underwent operation on the 11th-16th day of gestation. In each case the wall of the uterine horn was slit and the placentae together with the attached embryos were completely removed. The operation was completed by sewing up the incision on the uterine horn and that on the abdominal wall.

The corpora lutea in these cases showed a close similarity to those observed in the hysterectomized-pregnant animals. The average survival of the corpora lutea after operation was 10 days, 23.3 days being the average length of the total life span. The total life of the corpora lutea in this group of rabbits was about 3 days longer than that of the hysterectomized-pregnant animals. This is explicable as several of the latter group underwent operation at an early stage of gestation.

*Effect of oestrone injection on the corpora lutea of hysterectomized-pregnant rabbits*

We are at present ignorant of the factor present in the placenta which is essential for the maintenance of an active corpus luteum. It has become clear, however, that the corpus luteum is sensitive to oestrogenic stimulation. If the placenta in the rabbit does produce some oestrogen-like substance, the discrepancy in the behaviour of the corpora lutea in pregnant and pseudopregnant animals could be easily explained. It is therefore necessary to see whether the corpus luteum in pregnant rabbits is sensitive to the administration of oestrogen as it is in pseudopregnant animals.

Six pregnant rabbits were hysterectomized and given injections of oestrone. Three of them received a dose of 0.1 mg. daily for 10–15 days. Another two animals received 1 mg. daily for 10 days. The rest were injected with 2 mg. doses for the same period. The corpora lutea of the animals receiving 0.1 mg. doses appeared to be adequately maintained in one case only; those of the other two animals regressed during the injection. Animals receiving 1 mg. doses had very large and well-vascularized corpora lutea, which completely regressed about one week after stopping the injection. Corpora lutea of the animal receiving the highest doses of oestrone were very well maintained; they underwent complete regression about 10 days after stopping the injections (Table 6).

Table 6. *The survival period of the corpora lutea in hysterectomized-pregnant rabbits injected with oestrone*

Rabbit no.	Time of hysterectomy after coitus days	Dosage of oestrone mg./day	Injection period days	Life of corpora lutea days	Regression of corpora lutea after injection days
R21	12	0.1	10	32	10
R24	15	0.1	15	24	Regressed on 9th day of injection
R22	22	0.1	14	—	Disappeared at the end of injection
R60	9	1	10	—	Animal died on cessation of injection; corpora lutea very large and highly vascularized
R62	9	2	10	29	10
R76	14	1	10	31	7

#### DISCUSSION

The life of the corpora lutea is lengthened by removal of the uterus, and shortened by implantation of uterine tissue into the hysterectomized-pseudopregnant rabbits. These facts suggest that the uterine endometrium produces some substance that inhibits luteal persistence. But since the corpora lutea of the intact pseudopregnant rabbits could be maintained by adequate doses of oestrogen, an alternative explanation is available. As the function of the uterus and the maintenance of the corpus luteum both require oestrogen, it is conceivable that the removal of the uterus may spare some available oestrogen and leave it at the disposal of the corpora lutea, thus lengthening their survival. It is, therefore, expected that implantation of the uterus into hysterectomized-pseudopregnant animals would restore the normal survival of the corpora lutea.

The above explanation does not seem adequate to interpret the results found in the pregnant animals, because the removal of the uterus and uterine contents or extirpation of the placenta leaving the uterus intact leads to a precipitous atrophy of the corpora lutea while the implantation of the placenta in a hysterectomized-pregnant animal tends to postpone their regression. The factor concerned in regulating the corpus luteum under pregnant conditions appears to be present in the placental tissue; the uterus appears to play a less important part.

Careful analysis of the experimental results discloses several interesting points. First, the life of the corpora lutea in hysterectomized-pseudopregnant rabbits was about as long as in hysterectomized-pregnant rabbits with placental implants. The survival in the hysterectomized-pregnant animals was about as long as in the hysterectomized-pseudopregnant animals with uterine implants. Secondly, in the pregnant rabbit,

the presence or absence of the uterus has no effect on the activity of the corpus luteum; the precipitous atrophy of this structure following hysterectomy is obviously due to the removal of a luteal-stimulating substance of placental origin. Thirdly, corpora lutea in both pregnant and pseudopregnant states could be maintained by the administration of oestrogen; the regression of the corpora lutea in either of these two conditions is very likely effected by a decrease of oestrogen content. It is therefore inferred that the substance produced by the placental tissue is of the nature of an oestrogen. Taking all these facts into consideration, we believe that oestrogen may be the common factor for the maintenance of the corpus luteum in both pregnant and pseudopregnant conditions.

The present hypothesis is handicapped by the fact that the presence of oestrogenic hormones in the placental tissue of the rabbit has not been demonstrated. But the sustaining action of oestrogen on the corpus luteum has been well established, even in hypophysectomized animals and this is probably the only satisfactory explanation of our experimental results.

#### SUMMARY

The survival of the corpora lutea in pseudopregnant rabbits is prolonged by hysterectomy but may be restored to normal by implantation of uterine tissue. Oestrone injections into normal pseudopregnant animals sustain the function of the luteal tissue.

The removal of the uterus from a pregnant rabbit speeds the regression of the corpus luteum. Implantation of placenta or injection of oestrone into hysterectomized-pregnant animals prolongs the survival of this structure.

The significance of oestrogen in the maintenance of luteal function in both pregnant and pseudopregnant states has been discussed.

The writers are indebted to Prof. Chiao Tsai for his criticisms in the preparation of the manuscript. Oestrone was kindly provided by the Organon Laboratories, London, to whom our thanks are due.

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# LOGISTIC DOSE-RESPONSE CURVES: A THEORETICAL APPROACH

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(Received 26 April 1945)

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## NOTATION

$x$	Dose of hormone administered; daily dose where treatment is repeated.
$y, y_n$	Weight of test organ; weight after $n$ days.
$L$	Limiting weight of test organ.
$t$	Time.
$\alpha, \beta$	Constants of the logistic curve.
$a$	A growth coefficient.
$z$	Concentration of hormone in the relevant part of the body.
$n$	Number of injections.
$v$	Volume of injected solution or implanted tablet. Where this is not assumed constant, $v_0$ denotes the initial volume and $v$ the volume after time $t$ .
$l$	Threshold concentration of the hormone.
$A, A_n$	Amount of hormone removed from a depot.
$B$	Amount of hormone removed from circulation (by excretion or neutralization).
$C$	Constant of integration.
$\ln X \equiv \log_e X$ .	

(Since this paper is, in a sense, complementary to that of Emmens [1940], it was considered desirable to retain his notation as far as possible. It should be noted that this makes it necessary to treat  $x$  as a constant and  $A$  and  $B$  as variables in various differential equations.)

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## INTRODUCTION

Emmens [1940] has shown that a considerable variety of dose-response relations can be adequately represented by the logistic curve

$$y = \frac{L}{1 + e^{(\beta-x)/\alpha}}, \quad (1)$$

where  $x$  = dose,  $y$  = weight of test organ, and  $L$ ,  $\alpha$  and  $\beta$  are constants,  $L$  being the limiting weight of the organ. He found, further, that, with a constant dose, the time-response curve was logistic in some cases, i.e.

$$y = \frac{L}{1 + e^{(\beta-t)/\alpha}}, \quad (2)$$

where  $t$  = time. He considered that the logistic dose curve could be explained as a consequence of the time curve being logistic in certain cases (e.g. the response of rats' ovaries to various gonadotrophins), but this explanation proved inadequate in other cases (e.g. the response of the pigeon crop-gland to prolactin, and of the guinea-pig thyroid to thyrotrophin).

The purpose of the present communication is to examine the conditions under which a logistic dose-response curve may be expected on theoretical grounds, and to show that it does not necessarily depend on a logistic time-response curve.

## DERIVATION OF A DOSE LOGISTIC FROM A TIME LOGISTIC

The logistic growth (time-response) curve is obtained by integrating the equation

$$\frac{dy}{dt} = ay(L-y). \quad (3)$$

This assumes that the rate of growth of the organ varies jointly with (a) its size at the time in question, and (b) the defect from its maximum size. Assumption (a) is simply the 'compound interest' law of multiplication of cells, while (b) is the simplest assumption to account for the slowing down of growth as the maximum size is approached. By a suitable interpretation of the symbols, the same equation can be applied to the multiplication of micro-organisms in the presence of a limited supply of nutriment [M'Kendrick & Pai, 1911], the growth of human populations [Verhulst, 1844; Pearl, 1924] or the progress of auto-catalytic reactions [Ostwald, 1883].

Integration of (3) gives

$$\ln \frac{y}{L-y} - \ln \frac{y_0}{L-y_0} = aLt, \quad (4)$$

where  $y_0$  = initial weight of test organ. Equation (4) may be written in the exponential form

$$y = \frac{L}{1 + \frac{L-y_0}{y_0} e^{-aLt}}, \quad (5)$$

which is transformed into (2) by the substitutions

$$\alpha = 1/aL, \quad \beta = \alpha \ln \frac{L-y_0}{y_0}.$$

Now  $a$  is clearly a growth coefficient (see (3)) and will depend on the joint action of the injected substance and the animal's own hormones. The simplest assumption is that it is merely a linear function of the dose, i.e.

$$a = bx + c. \quad (6)$$

Here  $c$  may be regarded as a composite constant, allowing for the effect of intrinsic hormones (assumed constant) and the possible existence of a threshold dose.

Substituting for  $a$  in (5),

$$y = \frac{L}{1 + \frac{L-y_0}{y_0} e^{-(bx+c)Lt}} \quad (7)$$

If now  $x$  is varied keeping  $t$  constant, we obtain a logistic dose-response curve, for (7) reduces to (1) on substituting

$$\alpha = 1/bLt, \quad \beta = \alpha \ln \frac{L-y_0}{y_0} - \frac{b}{c}.$$

Emmens deduced the dose curve (1) by assuming the standard interval,  $\alpha$ , of the time curve (2) to be inversely proportional to the dose. This is equivalent to putting  $a = bx$ , ignoring the constant  $c$  in (6). This omission is of little consequence when massive doses are given. But equation (6) itself involves an undue simplification, since it assumes that  $a$  depends only on the *magnitude* of the dose. Actually, of course, the response depends on the *spacing* of subdoses, the *rate* of absorption, etc. This means that the dose factor is intimately associated with a time factor or factors. Equation (6) should therefore be written

$$a = f(x, t), \quad (6a)$$

the precise function being determined by experimental conditions. It will be found that (6a) leads to a logistic dose-response curve if  $f(x, t)$  is linear in  $x$ .

#### SOME GENERAL CONSIDERATIONS

Suppose that  $a = f(x, t)$  can be thrown into the form

$$a = \lambda x \phi(t) + \mu \psi(t) + \nu. \quad (8)^*$$

Equation (3) may then be written

$$dy/y(L-y) = (\lambda x \phi(t) + \mu \psi(t) + \nu) dt.$$

Integrating,

$$(\ln y - \ln(L-y))/L = \lambda x \Phi(t) + \mu \Psi(t) + \nu t + C,$$

where

$$\Phi(t) = \int \phi(t) dt \quad \text{and} \quad \Psi(t) = \int \psi(t) dt.$$

When  $t = 0$ ,  $y = y_0$ ,

$$\therefore (\ln y_0 - \ln(L-y_0))/L = \lambda x \Phi(0) + \mu \Psi(0) + C,$$

$$\therefore \left( \ln \frac{y}{L-y} - \ln \frac{y_0}{L-y_0} \right) / L = \lambda x (\Phi(t) - \Phi(0)) + \mu (\Psi(t) - \Psi(0)) + \nu t, \quad (9)$$

whence

$$y = \frac{L}{1 + \frac{L-y_0}{y_0} e^{-L(\lambda x(\Phi(t)-\Phi(0)) + \mu(\Psi(t)-\Psi(0)) + \nu t)}} \quad (10)$$

If  $t$  is kept constant, equation (10) becomes a logistic dose-response curve. On the other hand, with a constant dose, the time-response curve is, in general, more complex, reducing to the logistic (2) only in the special case where the index is linear in  $t$ . Thus, when  $a (= f(x, t))$  is linear in  $x$ , a logistic dose-response curve is obtained which does not depend on the time-response curve being logistic.

• The simpler formulation  $a = x\phi(t) + \psi(t)$  would adequately express the case where  $f(x, t)$  is linear in  $x$ . The formulation (8) is preferred for ease of comparison with expressions derived in specific cases later.

Some comments on the validity of (10) are necessary. In performing the integration,  $y$  was tacitly assumed to be a continuous function of  $t$ . We should expect this where only one dose is given. In the case of a series of doses, however, we should expect a spurt of growth, corresponding to each fresh dose, leading to a curve somewhat like Fig. 1.

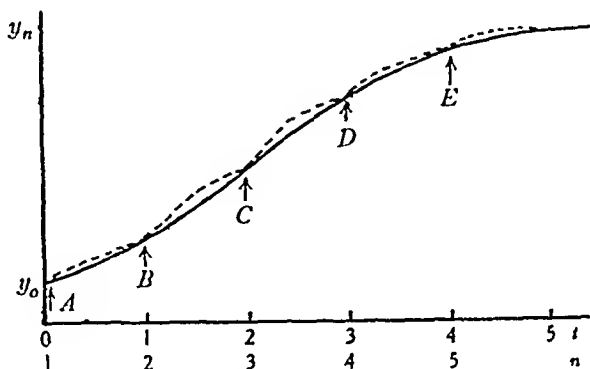


FIG. 1. The broken line represents diagrammatically the actual growth curve of the organ in response to doses at  $A, B, \dots, E$ . The continuous line is the 'smooth growth curve' (defined below).

Here the growth curve is continuous, but its slope ( $dy/dt$ ) is discontinuous. This implies that  $\phi(t)$  and  $\psi(t)$  are discontinuous functions—as they clearly must be if dosage is discontinuous. Their discontinuities are, however, finite in number and in magnitude, and under these conditions the functions are integrable. The formulation (9) or (10) is therefore valid when a series of doses is given. In this case, however,  $\phi(t)$  and  $\psi(t)$  will, in general, assume a more complex form after each successive dose, owing to the accumulation of residues of earlier doses, and it will usually be necessary to evaluate them for each of the intervals  $A$  to  $B$ ,  $B$  to  $C$ , etc., in turn. Suppose there are  $n$  daily doses (any other time interval could, of course, be taken) and let  $\phi_m(t)$ ,  $\psi_m(t)$  be the forms taken by  $\phi(t)$  and  $\psi(t)$  on the  $m$ th day. At the end of the  $n$ th day, equation (9) may be written

$$\left( \ln \frac{y_n}{L - y_n} - \ln \frac{y_0}{L - y_0} \right) / L = \lambda x \left\{ \sum_{m=1}^n (\Phi_m(1) - \Phi_m(0)) \right\} + \mu \left\{ \sum_{m=1}^n (\Psi_m(1) - \Psi_m(0)) \right\} + n\nu. \quad (9a)$$

If absorption and excretion of the substance are so rapid that each dose has virtually been eliminated by the time the next dose is given, the functions  $\phi_m(t)$  and  $\psi_m(t)$  will presumably be the same in each interval, and the position at the end of the  $n$ th day is given by

$$\left( \ln \frac{y_n}{L - y_n} - \ln \frac{y_0}{L - y_0} \right) / L = n \{ \lambda x [\Phi(1) - \Phi(0)] + \mu [\Psi(1) - \Psi(0)] + \nu \}. \quad (9b)$$

Now, in practice, we are not interested in the whole of the irregular growth curve, but only in selected points. The division of the total dose into a series of subdoses is a makeshift procedure, intended to approximate to continuous administration and so to imitate more closely the natural action of intrinsic hormones. Hence, in drawing the growth curve it is usual to smooth out the 'accidental' irregularities due to imperfect approximation to continuous dosage. This is generally done by measuring the test organ 24 hr. (or a multiple of this) after each dose when the doses are given daily, and

drawing a smooth curve through the resulting points. To bring theory into line with practice, we have to consider a smooth curve through the points  $A, B, C$ , etc. (Fig. 1). Such a curve will subsequently be called the 'smooth' growth curve. It may be formally defined as the theoretical curve through the extremities of the ordinates corresponding to the abscissae at the times at which the doses are given, and one time unit after the last dose.

In the case just discussed (equation (9b)), the points which define the smooth growth curve are determined by giving  $n$  the values  $1, 2, \dots, n$  in turn and evaluating  $y_n$  in each case. The smooth growth curve is therefore identical with the  $(y_n, n)$  curve, and is logistic.\* In the more general case (equation (9a)), we cannot say whether the smooth growth curve is logistic until the form of the functions  $\Phi_n(t)$  and  $\Psi_n(t)$  is known. If they are linear in  $n$  the curve will be logistic, but, in general, not otherwise.

We may note that, with the growth law assumed in this section, the final size of the organ will, in general, depend on the manner in which the total dose is subdivided. If, for instance,  $2n$  daily doses of  $\frac{1}{2}x$  are given instead of  $n$  daily doses of  $x$ , the right-hand side of (9b) becomes

$$2n\{\lambda \cdot \frac{1}{2}x[\Phi(1) - \Phi(0)] + \mu[\Psi(1) - \Psi(0)] + \nu\},$$

which is only identical with the original when  $\mu = \nu = 0$ .

The results obtained in this section may be summarized as follows. If the growth coefficient  $a$  is a linear function of the dose, then:

- (1) in the case of a single dose, the dose-response curve is logistic, but the time-response curve is, in general, more complex;
- (2) when the dose is divided into equal subdoses, evenly spaced, the dose-response curve is logistic. The 'smooth growth curve' (i.e. the theoretical curve corresponding to the time-response curve as usually plotted in practice) is, in general, more complex, but tends to a simple logistic form when absorption, action and elimination of each subdose are virtually complete by the time the next subdose is given.

The next step is to consider some specific cases to see whether an equation of the form (8) can be deduced on the basis of fairly plausible assumptions. Only the simpler cases will be dealt with. Oral administration will not be considered, since the complex and continually varying nature of the intestinal contents, and the probability that some of the substance will pass unchanged into the faeces make it unlikely that the absorption will follow any simple mathematical law. In the case of injections, it will be necessary to assume that the substance passes directly into the body fluids and tissues without being stored and subsequently released on its way to the site of action.

The growth law (3), i.e.  $dy/dt = ay(L - y)$ , will be assumed throughout, the coefficient  $a$  remaining to be determined in each case. Now  $a$  will not depend directly on the dose, but rather on the effective concentration of the hormone in the body fluids and tissues. The simplest assumption here is the linear relation

$$a = b(z - l), \quad (11)$$

where  $z$  = total concentration of the hormone in the relevant part of the body, and  $l$  = threshold concentration.

\* Actually,  $n$ , being the number of doses, is necessarily integral, and the  $(y_n, n)$  'curve' consists of a series of isolated points. These points, however, lie on a logistic, which is therefore the best-fitting smooth growth curve.



## SUBCUTANEOUS OR INTRAMUSCULAR INJECTION

(a) *Volume of depot assumed constant*

Consider first the case of a single dose  $x$  dissolved or suspended in a volume  $v$ , and assume that the conditions are such that, to a first approximation, the volume of the 'depot' remains constant during the experimental period. Let  $A$  be the amount of hormone absorbed from the depot, and  $B$  the amount removed from circulation (by excretion or neutralization) after time  $t$ . Suppose the rate of secretion of the hormone in question by the animal's own glands is constant and  $= h$  in unit time. Then the amount of the hormone present in the body tissues and fluids after time  $t$  is

$$H + A + ht - B,$$

where  $H$  is the amount present at the beginning of the period; the concentration in the relevant fluids or tissues is given by

$$z = k(H + A + kt - B). \quad (12)$$

Assume, further, that the rate of absorption of hormone from the depot is proportional to its concentration in the depot, and that the rate of removal from circulation is proportional to its concentration in the body, i.e.

$$\frac{dA}{dt} = k_1 \frac{x - A}{v} \quad (13)$$

$$\text{and} \quad \frac{dB}{dt} = k_2 z. \quad (14)$$

$$\text{From (13),} \quad x - A = x e^{-k_1 t/v}, \quad (15)$$

the integration constant being determined from the initial condition that  $A = 0$  when  $t = 0$ .

Differentiating (12),

$$\begin{aligned} \frac{dz}{dt} &= k \left( \frac{dA}{dt} + h - \frac{dB}{dt} \right) \\ &= k \left( \frac{k_1}{v} x e^{-k_1 t/v} + h - k_2 z \right), \end{aligned}$$

by virtue of (13), (14) and (15). This equation can be integrated after transposing and multiplying throughout by  $e^{kk_2 t}$ , and gives

$$z e^{kk_2 t} = \frac{k k_1 x}{v} \frac{e^{(kk_2 - k_1/v)t}}{kk_2 - k_1/v} + k h \frac{e^{kk_2 t}}{k k_2} + C.$$

When  $t = 0$ ,  $z = kH$ ,

$$\therefore C = kH - h/k_2 - k k_1 x / (k k_2 v - k_1),$$

$$\text{whence} \quad z = \frac{k k_1 x}{k k_2 v - k_1} (e^{-k_1 t/v} - e^{-kk_2 t}) + \left( kH - \frac{h}{k_2} \right) e^{-kk_2 t} + \frac{h}{k_2}, \quad (16)$$

and, replacing groups of constants by new composite constants,

$$a = b(z - l) = \lambda x (e^{-pt} - e^{-qt}) + \mu e^{-qt} + v. \quad (17)$$

This is linear in  $x$  but not in  $t$ ; hence the dose-response curve is logistic, but not the time-response curve.

It follows from the preceding section (p. 401) that if  $n$  daily injections are given, each equal to  $x$ , and the experiment ends 24 hr. after the last injection, the dose-response curve will again be logistic. If the residual injectate after 24 hr. is negligible, the smooth growth curve is (by (9b)) the  $(y_n, n)$  curve:

$$\left( \ln \frac{y_n}{L-y_n} - \ln \frac{y_0}{L-y_0} \right) / L = n \left\{ \lambda x \left[ \frac{1}{p} (1-e^{-p}) - \frac{1}{q} (1-e^{-q}) \right] + \frac{\mu}{q} (1-e^{-q}) + \nu \right\}, \quad (18)$$

and is logistic. It remains to determine the smooth growth curve when absorption and elimination are less rapid, and residues of earlier doses are not negligible.

By (16), at the end of the first day ( $t = 1$ ),

$$\begin{aligned} z &= \frac{kk_1x}{kk_2v-k_1} (e^{-k_1/t} - e^{-kk_2}) + \left( kH - \frac{h}{k_2} \right) e^{-kk_2} + \frac{h}{k_2} \\ &= kH_1, \text{ the initial concentration on the second day.} \end{aligned} \quad (19)$$

By (15), the amount of hormone remaining in the first depot is  $xe^{-k_1/t}$ . A second injection is now given. Let the amounts of hormone removed from the two depots after time  $t$  (measured from the second injection) be  $A_1$  and  $A_2$ . Then

$$\frac{dA_1}{dt} = k_1 \frac{xe^{-k_1/t} - A_1}{v},$$

$$\frac{dA_2}{dt} = k_1 \frac{x - A_2}{v},$$

giving

$$xe^{-k_1/t} - A_1 = xe^{-k_1(t+1)/v}$$

and

$$x - A_2 = xe^{-k_1/tv}.$$

We have

$$z = k(H_1 + A_1 + A_2 + ht - B),$$

whence

$$\frac{dz}{dt} = k \left\{ \frac{k_1x}{v} (e^{-k_1(t+1)/v} + e^{-k_1/tv}) + h - k_2z \right\}.$$

Integrating as before, and evaluating the integration constant from the condition that, when  $t = 0$ ,  $z = kH_1$ , given by (19),

$$z = \frac{kk_1x}{kk_2v-k_1} (e^{-k_1(t+1)/v} + e^{-k_1/tv} - e^{-kk_2(t+1)} - e^{-kk_2t}) + \left( kH - \frac{h}{k_2} \right) e^{-kk_2(t+1)} + \frac{h}{k_2}.$$

Putting  $t = 1$  we obtain the initial concentration for the third day. Immediately after the third injection the depots will contain  $xe^{-2k_1/t}$ ,  $xe^{-k_1/t}$  and  $x$  respectively, and  $z$  can be evaluated as above.

In general, it may be shown inductively that after  $n$  injections,

$$z = \frac{kk_1x}{kk_2v-k_1} \left\{ \sum_{m=0}^{n-1} (e^{-k_1(t+m)/v} - e^{-kk_2(t+m)}) \right\} + \left( kH - \frac{h}{k_2} \right) e^{-kk_2(t+n-1)} + \frac{h}{k_2}, \quad (20)$$

and we may write (cf. (16) and (17))

$$\begin{aligned} \alpha &= \lambda x \left\{ \sum_{m=0}^{n-1} (e^{-p(t+m)} - e^{-q(t+m)}) \right\} + \mu e^{-q(t+n-1)} + \nu \\ &= \lambda x \left\{ \frac{1-e^{-np}}{1-e^{-p}} e^{-pt} - \frac{1-e^{-nq}}{1-e^{-q}} e^{-qt} \right\} + \mu e^{-q(t+n-1)} + \nu. \end{aligned}$$

With the notation of (9a),

$$\Phi_m(t) = -\frac{1}{p}e^{-pt}\frac{1-e^{-mp}}{1-e^{-p}} + \frac{1}{q}e^{-qt}\frac{1-e^{-mq}}{1-e^{-q}}$$

and

$$\Psi_m(t) = -\frac{1}{q}e^{-q(t+m-1)},$$

whence

$$\Phi_m(1) - \Phi_m(0) = \frac{1}{p}(1 - e^{-mp}) - \frac{1}{q}(1 - e^{-mq}) \quad (20a)$$

and

$$\Psi_m(1) - \Psi_m(0) = \frac{1}{q}(e^{-(m-1)q} - e^{-mq}).$$

Hence, by (9a),

$$\begin{aligned} & \left( \ln \frac{y_n}{L-y_n} - \ln \frac{y_0}{L-y_0} \right) / L \\ &= \lambda x \left\{ \frac{1}{p} \left( n - e^{-p} \frac{1 - e^{-np}}{1 - e^{-p}} \right) - \frac{1}{q} \left( n - e^{-q} \frac{1 - e^{-nq}}{1 - e^{-q}} \right) \right\} + \frac{\mu}{q}(1 - e^{-nq}) + n\nu. \end{aligned} \quad (21)$$

Here  $n$  appears not only linearly, but also as an exponential function; hence the smooth growth curve is not a logistic, though the dose-response curve obviously is.

If  $p$  and  $q$  are large,  $e^{-p}$  and  $e^{-q}$  are very small, and, to a first approximation, negligible. In this case, the right-hand side of (21) reduces to  $n\lambda x(1/p - 1/q) + \mu/q + n\nu$ , which, being linear in  $n$ , leads to a logistic smooth growth curve. But  $p$  and  $q$  are large when  $k_1$  and  $k_2$  are large, that is, when absorption and elimination are rapid. Thus we reach a previous conclusion by a different route. In fact, if  $e^{-p}$  and  $e^{-q}$  are negligible, equations (18) and (21) become identical, as would be expected if the corresponding arguments are valid.

Equation (21) also becomes approximately linear in  $n$  if  $n$  is large, even if  $p$  and  $q$  are not large.

#### (b) Volume of depot not constant

So far, changes in volume of the injectate have been assumed to be negligible. This approximation is obviously of limited validity. In practice, the injectate will usually be a dilute solution, hence removal of the *solute* will not appreciably affect the volume.

The assumption of constant volume is perhaps most justifiable when the hormone is of high molecular weight and dissolved in isotonic saline. A depot containing a non-isotonic aqueous solution would presumably undergo fairly rapid volume change. If an oily medium is used, the approximation will be valid if the hormone is absorbed sufficiently rapidly compared with the medium. This is perhaps most likely when the solvent is an inert substance, not too similar chemically to the body fats. It is worth noting, however, that if the medium is being removed by virtue of its solubility in the body fats, the residual medium will almost certainly be absorbing some fat at the same time, thus retarding the change of volume. Hence, conclusions based on the assumption of constant volume may well have a wider application in practice than would be expected at first sight.

If volume changes cannot be neglected, the problem becomes very difficult, and a solution of general validity may be impossible. The rate of absorption of both solvent and solute will presumably depend on the area in contact with the tissues, while the absorption of solute will also depend on concentration. Thus changes in area as well as volume of the injectate have to be considered, and it is necessary to postulate a

relation between them, e.g. area  $\propto$  (volume)<sup>3</sup>. But here the proportionality 'constant' will depend on the shape of the depot, and will only remain a constant if the shape is retained. In practice, owing to uneven pressure of the tissues, the shape is likely to be irregular, and any appreciable change in volume may involve a change in shape (e.g. the injectate may break into two separate drops). Such changes will depend on the exact site of injection, the plumpness of the animal, etc. Thus agreement between experimental results and a theoretical curve may involve a strong element of chance.

Ignoring this possible complication, let us assume that the rate of absorption of the solvent is proportional to the surface area of the depot, which in turn is proportional to (volume)<sup>3</sup>. If the solution is dilute, volume changes due to removal of solute may be neglected, and we may write

$$\frac{dv}{dt} = k_3 v^{\frac{1}{3}}. \quad (22)$$

For a decreasing volume,  $k_3$  is negative. Now, if the solvent is removed rapidly compared with the solute, a point will be reached where the solution can no longer be considered dilute. It will therefore be assumed that  $|k_3|$  is not large compared with  $k'_1$ , the constant relating to the removal of solute.\*

The solution of (22) is

$$3(v^{\frac{1}{3}} - v_0^{\frac{1}{3}}) = k_3 t \quad (v_0 = \text{initial volume}). \quad (23)$$

The rate of removal of the solute will, presumably, vary jointly with its concentration and the surface area of the depot, i.e.

$$\begin{aligned} \frac{dA}{dt} &= k'_1 \frac{x-A}{v} v^{\frac{1}{3}} = k'_1 \frac{x-A}{v^{\frac{2}{3}}} \\ &= k'_1 \frac{x-A}{\frac{1}{3}k_3 t + v_0^{\frac{1}{3}}} \quad (\text{by (23)}). \end{aligned} \quad (24)$$

Integrating, with the initial condition  $A = 0$  when  $t = 0$ ,

$$\ln \frac{x-A}{x} = \frac{3k'_1 \ln \frac{v_0^{\frac{1}{3}}}{\frac{1}{3}k_3 t + v_0^{\frac{1}{3}}}}{k_3},$$

or

$$x-A = x \left(1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t\right)^{-3k'_1/k_3}. \quad (25)$$

As before,  $\frac{dz}{dt} = k \left( \frac{dA}{dt} + h - \frac{dB}{dt} \right)$

$$= k \left( \frac{k'_1 x}{v_0^{\frac{1}{3}}} \left(1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t\right)^{-(1+3k'_1/k_3)} + h - k_2 z \right) \quad (\text{by (24) and (25)}),$$

whence 
$$z e^{k k_2 t} = \frac{k k'_1 x}{v_0^{\frac{1}{3}}} \int e^{k k_2 t} \left(1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t\right)^{-(1+3k'_1/k_3)} dt + k h \frac{e^{k k_2 t}}{k k_2} + C.$$

In general, the above integral cannot be evaluated in a finite number of terms. Denoting it by  $F_1(t)$  and evaluating  $C$  from the condition  $z = kH$  when  $t = 0$ ,

$$z = \frac{k k'_1 x}{v_0^{\frac{1}{3}}} e^{-k k_2 t} \{F_1(t) - F_1(0)\} + \left(kH - \frac{h}{k_2}\right) e^{-k k_2 t} + \frac{h}{k_2}. \quad (26)$$

At the end of the first day ( $t = 1$ )

$$\begin{aligned} z &= \frac{k k'_1 x}{v_0^{\frac{1}{3}}} e^{-k k_2} \{F_1(1) - F_1(0)\} + \left(kH - \frac{h}{k_2}\right) e^{-k k_2} + \frac{h}{k_2} \\ &= kH_1, \text{ the initial concentration on the second day.} \end{aligned} \quad (27)$$

\* As noted above, if  $|k_3|$  is sufficiently small compared with  $k'_1$  the volume may be assumed constant.

Suppose a second injection is now given. During the second day, (23) will become  $3(v^{\frac{1}{3}} - v_1^{\frac{1}{3}}) = k_3 t$ , where  $t$  is measured from the time of the second injection and  $v_1$  is given by putting  $v = 1$  in (23).

$$\text{Hence} \quad v^{\frac{1}{3}} = \frac{k_3}{3}(t+1) + v_0^{\frac{1}{3}}. \quad (28)$$

At the time of the second injection, the first depot contains (by (25))  $x(1 + k_3/3v_0^{\frac{1}{3}})^{-3k'_1/k_3}$  of the hormone. The rate of removal of hormone from this depot during the second day is therefore

$$\begin{aligned} \frac{dA_1}{dt} &= \frac{k'_1}{v^{\frac{1}{3}}} \{x(1 + k_3/3v_0^{\frac{1}{3}})^{-3k'_1/k_3} - A_1\} \\ &= k'_1 \frac{x(1 + k_3/3v_0^{\frac{1}{3}})^{-3k'_1/k_3} - A_1}{\frac{1}{3}k_3(t+1) + v_0^{\frac{1}{3}}} \quad (\text{by (28)}), \end{aligned}$$

with the initial condition  $A_1 = 0$  when  $t = 0$ , whence

$$x(1 + k_3/3v_0^{\frac{1}{3}})^{-3k'_1/k_3} - A_1 = x \left\{ 1 + \frac{k_3}{3v_0^{\frac{1}{3}}}(t+1) \right\}^{-3k'_1/k_3}.$$

The rate of removal of hormone from the second depot will be the same as that relating to the first depot on the first day, i.e.

$$\frac{dA_2}{dt} = \frac{k'_1 x}{v_0^{\frac{1}{3}}} \left( 1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t \right)^{-(1+3k'_1/k_3)} \quad (\text{by (24) and (25)}).$$

Substituting for  $dz/dt$  and integrating,

$$\begin{aligned} ze^{kk_2 t} &= \frac{kk'_1 x}{v_0^{\frac{1}{3}}} \int e^{kk_2 t} \left( 1 + \frac{k_3}{3v_0^{\frac{1}{3}}}(t+1) \right)^{-(1+3k'_1/k_3)} dt \\ &\quad + \frac{kk'_1 x}{v_0^{\frac{1}{3}}} \int e^{kk_2 t} \left( 1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t \right)^{-(1+3k'_1/k_3)} dt + \frac{h}{k_2} e^{kk_2 t} + C \\ &= \frac{kk'_1 x}{v_0^{\frac{1}{3}}} \{F_2(t) + F_1(t)\} + \frac{h}{k_2} e^{kk_2 t} + C. \end{aligned}$$

When  $t = 0$ ,  $z (= kH_1)$  is given by (27), and substituting the value of  $C$  so found,

$$\begin{aligned} z &= \frac{kk'_1 x e^{-kk_2 t}}{v_0^{\frac{1}{3}}} \{F_1(t) + F_2(t) - F_1(0) - F_2(0)\} + \frac{kk'_1 x e^{-kk_2(t+1)}}{v_0^{\frac{1}{3}}} - \{F_1(1) - F_1(0)\} \\ &\quad + \left( kH - \frac{h}{k_2} \right) e^{-kk_2(t+1)} + \frac{h}{k_2}. \end{aligned}$$

Putting  $t = 1$ , we obtain the initial concentration for the third day. The argument can be repeated, and it can be shown inductively that, on the  $n$ th day,

$$\begin{aligned} z &= kk'_1 x v_0^{-\frac{1}{3}} \left\{ e^{-kk_2 t} \sum_{r=1}^n (F_r(t) - F_r(0)) + e^{-kk_2(t+1)} \sum_{r=1}^{n-1} (F_r(1) - F_r(0)) \right. \\ &\quad + e^{-kk_2(t+2)} \sum_{r=1}^{n-2} (F_r(1) - F_r(0)) + \dots + e^{-kk_2(t+n-2)} \sum_{r=1}^2 (F_r(1) - F_r(0)) \\ &\quad \left. + e^{-kk_2(t+n-1)} (F_1(1) - F_1(0)) \right\} + \left( kH - \frac{h}{k_2} \right) e^{-kk_2(t+n-1)} + \frac{h}{k_2}. \quad (29) \end{aligned}$$

Evidently  $a (= b(z-l))$  is of the form  $\lambda x\phi(t) + \mu\psi(t) + \nu$ . Now the terms denoted  $\mu\psi(t)$  and  $\nu$  are similar in form to the corresponding terms in the last section (cf. (20)), and their contribution to the  $(y_n, n)$  curve is therefore known. It remains to determine the contribution of  $\phi(t)$ .

$F_r(t)$  denotes

$$\int e^{k_k t} \left\{ 1 + \frac{k_3}{3v_0^2} (t+r-1) \right\}^{-(1+3k_i/k_j)} dt,$$

which may be written, for simplicity,

$$\int e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt \quad \text{or} \quad \int f_r(t) dt,$$

$$\begin{aligned} \phi_n(t) &= e^{-\alpha t} \sum_{r=1}^n \int_0^t f_r(t) dt + e^{-\alpha(t+1)} \sum_{r=1}^{n-1} \int_0^1 f_r(t) dt + e^{-\alpha(t+2)} \sum_{r=1}^{n-2} \int_0^1 f_r(t) dt \\ &\quad + \dots + e^{-\alpha(t+n-1)} \int_0^1 f_1(t) dt \\ &= e^{-\alpha t} \sum_{r=1}^n \int_0^t e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt + \frac{e^{-\alpha(t+1)} \sum_{r=1}^{n-1} \left\{ (1 - e^{-\alpha(n-r)}) \int_0^1 e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt \right\}}{1 - e^{-\alpha}} \end{aligned} \quad (30)$$

(by collecting like integral terms and summing the geometrical series of exponential multipliers), and

$$\Phi_n(t) = \int_0^1 \phi_n(t) dt.$$

$$\begin{aligned} \text{Now} \quad \int_0^1 e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt &= \int_{r-1}^r e^{\alpha(u-r+1)} (1 + \kappa u)^s du \quad (u = t+r-1) \\ &= e^{-\alpha(r-1)} \int_{r-1}^r e^{\alpha t} (1 + \kappa t)^s dt, \end{aligned} \quad (31)$$

since the variable does not appear in the definite integral, and

$$\begin{aligned} &\int_0^1 \frac{e^{-\alpha(t+1)}}{1 - e^{-\alpha}} \left\{ (1 - e^{-\alpha(n-r)}) \int_0^1 e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt \right\} dt \\ &= \frac{e^{-\alpha} - e^{-2\alpha}}{q(1 - e^{-\alpha})} \left\{ (1 - e^{-\alpha(n-r)}) e^{-\alpha(r-1)} \int_{r-1}^r e^{\alpha t} (1 + \kappa t)^s dt \right\} \\ &= \frac{e^{-\alpha q} - e^{-n\alpha}}{q} \int_{r-1}^r e^{\alpha t} (1 + \kappa t)^s dt. \end{aligned} \quad (32)$$

Also, integrating by parts,

$$\begin{aligned} &\int_0^1 e^{-\alpha t} \left\{ \int_0^t e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt \right\} dt \\ &= \left[ \frac{e^{-\alpha t}}{-\alpha} \int_0^t e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt - \int -\frac{1}{q} \{1 + \kappa(t+r-1)\}^s dt \right]_0^1 \\ &= \frac{1}{q} \int_0^1 \{1 + \kappa(t+r-1)\}^s dt - \frac{e^{-\alpha}}{q} \int_0^1 e^{\alpha t} \{1 + \kappa(t+r-1)\}^s dt \\ &= (\text{by (31)}) \quad \frac{1 \{1 + \kappa r\}^{s+1} - \{1 + \kappa(r-1)\}^{s+1}}{q\kappa(s+1)} - \frac{e^{-\alpha q}}{q} \int_{r-1}^r e^{\alpha t} (1 + \kappa t)^s dt. \end{aligned} \quad (33)$$

Hence, integrating equation (30), and using (32) and (33),

$$\begin{aligned} \Phi_n(t) &= \sum_{r=1}^n \frac{\{1 + \kappa r\}^{s+1} - \{1 + \kappa(r-1)\}^{s+1}}{q\kappa(s+1)} - \sum_{r=1}^n \frac{e^{-\alpha q}}{q} \int_{r-1}^r e^{\alpha t} (1 + \kappa t)^s dt \\ &\quad + \sum_{r=1}^{n-1} \frac{e^{-\alpha q} - e^{-n\alpha}}{q} \int_{r-1}^r e^{\alpha t} (1 + \kappa t)^s dt \\ &= \frac{(1 + \kappa n)^{s+1} - 1}{q\kappa(s+1)} - \frac{e^{-n\alpha}}{q} \int_0^n e^{\alpha t} (1 + \kappa t)^s dt, \end{aligned} \quad (34)$$

and finally ((9), (29), cf. (20), and (34)),

$$\frac{1}{L} \left( \ln \frac{y_n}{L-y_n} - \ln \frac{y_0}{L-y_0} \right) = \lambda x \left\{ \frac{\sum_{m=1}^n (1+\kappa m)^{s+1-n}}{q\kappa(s+1)} - \sum_{m=1}^n \frac{e^{-m\alpha}}{q} \int_0^m e^{qt}(1+\kappa t)^s dt \right\} + \frac{\mu}{q} (1-e^{-n\alpha}) + n\nu. \quad (35)$$

Now if, as is most likely, the volume of the depot decreases,  $k_3$  and therefore  $\kappa$  will be negative. Also  $\kappa$  will probably be numerically small; hence, unless  $n$  is very large,  $n\kappa$  may be taken to lie between 0 and 1. Under these conditions, the integrals in (35) can be expanded in terms of incomplete Beta functions by the following method, for which I am indebted to Dr J. O. Irwin.

Replacing  $\kappa$  by  $-c$  ( $c$  positive), and expanding the exponential term,

$$\begin{aligned} \int_0^m e^{qt}(1+\kappa t)^s dt &= \sum_{r=0}^{\infty} \frac{q^r}{r!} \int_0^m t^r (1-ct)^s dt \\ &= \sum_{r=0}^{\infty} \frac{q^r}{r!} \int_0^{cm} \frac{u^r}{c^{r+1}} (1-u)^s du \quad (\text{where } u=ct) \\ &= \frac{1}{c} \sum_{r=0}^{\infty} \left( \frac{q}{c} \right)^r \frac{1}{r!} B_{cm}(r+1, s+1) \\ &= \frac{1}{c} \sum_{r=0}^{\infty} \left( \frac{q}{c} \right)^r \frac{1}{r!} \frac{B_{cm}(r+1, s+1)}{B(r+1, s+1)} \frac{\Gamma(r+1)\Gamma(s+1)}{\Gamma(r+s+2)} \\ &= \frac{1}{c} \sum_{r=0}^{\infty} \left( \frac{q}{c} \right)^r \frac{\Gamma(s+1)}{\Gamma(r+s+2)} I_{cm}(r+1, s+1) \\ &= \frac{1}{c} \sum_{r=0}^{\infty} \left( \frac{q}{c} \right)^r \frac{I_{cm}(r+1, s+1)}{(r+s+2)(r+s+1)\dots(s+1)}. \end{aligned}$$

The functions  $I_{cm}(r+1, s+1)$  have been tabulated (*Tables of Incomplete Beta Functions*) and always lie between 0 and 1. The series is clearly convergent.

Reverting to equation (34), and writing it in full,

$$\Phi_n(t) = \frac{(1+nk_3/3v_0^{\frac{1}{3}})^{-3k'_1/k_3}-1}{kk_2(-3k'_1/k_3)(k_3/3v_0^{\frac{1}{3}})} - \frac{e^{-nk_2}}{kk_2} \int_0^n e^{kk_2 t} \left( 1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t \right)^{-(3k'_1/k_3+1)} dt.$$

Now

$$\lim_{k_3 \rightarrow 0} (1+nk_3/3v_0^{\frac{1}{3}})^{-3k'_1/k_3} = e^{-nk'_1/r_1^{\frac{1}{3}}},$$

and

$$\lim_{k_3 \rightarrow 0} \left( 1 + \frac{k_3}{3v_0^{\frac{1}{3}}} t \right)^{-(3k'_1/k_3+1)} = e^{-k'_1 t/r_1^{\frac{1}{3}}}.$$

Hence in the limit, when absorption of solvent is infinitely slow,

$$\Phi_n(t) = -v_0^{\frac{1}{3}} \frac{e^{-nk'_1/r_1^{\frac{1}{3}}}-1}{kk'_1k_2} - \frac{e^{-nk_2}}{kk_2} \int_0^n e^{(kk_2-k'_1/r_1^{\frac{1}{3}})t} dt$$

and by (29),

$$\lambda x \Phi_n(t) = \frac{kk'_1bx}{kk_2v_0^{\frac{1}{3}}-k'_1} \left\{ \frac{v_0^{\frac{1}{3}}}{k'_1} (1-e^{-nk'_1/r_1^{\frac{1}{3}}}) - \frac{1}{kk_2} (1-e^{-nk_2}) \right\}. \quad (36)$$

The corresponding expression in the last section (no volume change) was, by (20) and (20a) (*in extenso*),

$$\lambda x \Phi_n(t) = \frac{kk'_1bx}{kk_2v-k_1} \left\{ \frac{v}{k_1} (1-e^{-nk_1/r}) - \frac{1}{kk_2} (1-e^{-nk_2}) \right\}, \quad (37)$$

and  $v$  (in (37))  $\equiv v_0$  (in (36)). Comparing (13) and (24) ( $k_3 \rightarrow 0$ ), it is clear that  $k_1/v$  in the former corresponds to  $k'_1/v_0^{\frac{1}{2}}$  in the latter, and hence (36) is simply (37) in slightly different notation. As already noted, the terms denoted  $\mu\psi(t)$  and  $\nu$  are of the same form in both sections. We can thus reach the final equation (21) of the last section as a limiting case of (35) in this. The agreement affords a useful check.

## INTRAVENOUS INJECTION

If a dose  $x$  is injected intravenously, the concentration  $z$  will rapidly reach the value  $k(x+H)$  which may therefore be taken as the initial concentration. After a time  $t$ ,

$$z = k(x+H+ht-B),$$

whence

$$\frac{dz}{dt} = k(h-k_2z)$$

and

$$z = \frac{h}{k_2} + k \left( x+H - \frac{h}{k_2} \right) e^{-k_2 t}. \quad (38)$$

Here again,  $a$  is a linear function of  $x$ . In this case, rate of absorption does not enter, the maximum concentration of hormone being reached almost instantaneously. Elimination is therefore likely to be relatively rapid, and a logistic smooth growth curve may reasonably be expected.

If, however, elimination is slow, the value of  $a$  on the  $n$ th day can be calculated step-wise from (38) as in the preceding cases, or the same result may be obtained more simply as a limiting case of a preceding formulation, by regarding intravenous injection as equivalent to subcutaneous injection with infinitely rapid absorption. When  $k_1 \rightarrow \infty$  (p. 404),  $p \rightarrow \infty$  and  $e^{-p} \rightarrow 0$ , and (21) reduces to

$$\frac{1}{L} \left( \ln \frac{y_n}{L-y_n} - \ln \frac{y_0}{L-y_0} \right) = -\frac{\lambda x}{q} \left( n - e^{-q} \frac{1-e^{-nq}}{1-e^{-q}} \right) + \frac{\mu}{q} (1-e^{-nq}) + n\nu.$$

The  $(y_n, n)$  curve is clearly not logistic.

## UNIFORM CONTINUOUS DOSAGE

In the preceding sections it has been assumed that the relevant intrinsic hormone is secreted at a uniform rate. Uniform continuous administration of the extrinsic hormone is generally considered desirable and is approximated in practice by dividing the dose or by implanting a tablet of the hormone. It is therefore of interest to examine the hypothetical case in which the aim is completely achieved.

Here  $x$  will denote the daily dose, administered at a constant rate.

$$z = k\{H + (x+h)t - B\}$$

and

$$\frac{dz}{dt} = k(x+h-k_2z),$$

whence, with the condition  $z = kH$  when  $t = 0$ ,

$$z = \frac{x+h}{k_2} (1 - e^{-k_2 t}) + kHe^{-k_2 t}. \quad (39)$$

Again, the dose-response curve is logistic, but not the time-response curve. The question of determining the 'smooth growth curve' does not arise: it is identical with the actual growth curve, since there are no abrupt changes in slope due to discontinuous dosage.



If the rate of elimination is high,  $k_2$  is large and (39) approximates to  $z = (x + h)/k_2$ . This would make  $a$  linear in  $x$  and independent of  $t$ , leading to a logistic time-response curve. The same result follows when  $t$  is large; hence, in a long experiment, the growth curve will continually tend to a logistic form.

As a special case, *on the assumptions made*, the natural growth curve of the organ is not logistic, but approximates to a logistic if elimination of the relevant hormone is sufficiently rapid, or when secretion and excretion approach equilibrium, for then  $kH \rightarrow h/k_2$  and, with  $x = 0$ , (39) becomes  $z = h/k_2$ . The growth curve may, therefore, prove logistic within the limits of experimental error.

In the last paragraph it was implicitly assumed that body growth as a whole had virtually ceased, or was slow compared with the growth of the organ in question. Similar conclusions would follow if the hormone output keeps pace with the growth of the body. A constant concentration of hormone would lead to logistic growth of the organ. In general, however, none of these conditions can be assumed without direct experimental evidence.

#### IMPLANTATION OF A TABLET

If the hormone is implanted as a large tablet, the removal of a few milligrams will not appreciably affect its dimensions, and the rate of absorption, which will presumably vary as the area of the tablet, will remain practically constant for some time. The case is then appropriately treated as uniform continuous dosage as in the preceding section. When the tablet is small, however, or is left in position for a long time, the rate of absorption will presumably fall off as the surface diminishes. Experience shows that an implanted tablet usually retains its shape; hence, to a first approximation, we may assume that surface area  $\propto$  (volume) <sup>$\frac{1}{3}$</sup> .

Let  $v_0$  be the original volume of the tablet, and  $v$  the volume after time  $t$ .

Amount absorbed =  $A = v_0 - v$  and

$$\frac{dA}{dt} = -\frac{dv}{dt} = k_1 v^{\frac{1}{3}}, \quad (40)$$

whence, with the initial condition  $v = v_0$  when  $t = 0$ ,

$$3(v_0^{\frac{1}{3}} - v^{\frac{1}{3}}) = k_1 t. \quad (41)$$

Now 
$$z = k(H + A + ht - B),$$
 and 
$$\frac{dz}{dt} = k(k_1 v^{\frac{1}{3}} + h - k_2 z) = k\left(k_1\left(v_0^{\frac{1}{3}} - \frac{k_1 t}{3}\right)^2 + h - k_2 z\right) \quad \text{by (40) and (41).}$$

Integrating,

$$z = \frac{k_1}{k_2}\left(v_0^{\frac{1}{3}} - \frac{k_1 t}{3}\right)^2 + \frac{2k_1^2}{3kk_2^2}\left(v_0^{\frac{1}{3}} - \frac{k_1 t}{3}\right) + \frac{2k_1^3}{9kk_2^3} + \frac{h}{k_2} + Ce^{-kk_2 t}.$$

Evaluating the constant from the condition  $z = kH$  when  $t = 0$ ,

$$z = \left(\frac{k_1 v_0^{\frac{1}{3}} + h}{k_2} + \frac{2k_1^2 v_0^{\frac{1}{3}}}{3kk_2^2} + \frac{2k_1^3}{9kk_2^3}\right)(1 - e^{-kk_2 t}) - \left(\frac{2k_1^2 v_0^{\frac{1}{3}}}{3k_2} + \frac{2k_1^3}{9kk_2^2}\right)t + \frac{k_1^3}{9k_2} t^2 + kHe^{-kk_2 t}. \quad (42)$$

If now we substitute  $a = b(z - l)$  in (3) and integrate, it is obvious from an inspection of the right-hand side of (42) that the resulting expression will not be linear in  $t$ ; hence the growth curve will not be logistic. Also  $a$  is not linear in  $v_0$ ; hence the relation of response to the original size of tablet is not logistic.

*Note.* In practice, the absorption of tablets is apt to be a less simple process than has been assumed. The tablet frequently becomes encapsulated after a time, and this may retard absorption. A more curious observation is the penetration of body proteins into the tablet in the form of a hollow shell which remains behind when the original tablet material is extracted with a solvent [Folley, 1942]. This observation throws doubt on all previous studies of the rate of absorption of tablets.

It may be noted that when a compact solid passes into solution, the process consists of two stages: (a) the rapid formation of a layer of saturated solution around the solid; and (b) the relatively slow diffusion of solute from this layer into the outer liquid. The latter step determines the rate of the whole process. If circumstances favour the formation of an appreciable saturated layer, the effective surface will be increased. This may partly explain the finding of Emmens [1941] that the rate of absorption of certain tablets falls off less rapidly than would be expected as the size decreases. On the other hand, this result may be fictitious, the loss in weight of the tablets in the early stages being partly compensated by infiltration of protein.

#### DISCUSSION

The preceding treatment of dose-response and time-response curves may appear highly academic, since the equations involve a number of constants, the experimental evaluation of which would, at present, be impracticable. The primary object of the enquiry, however, was the *general* form of the equations. Experimental results have already been fitted to logistic curves. The above arguments suggest that this choice is, in many cases, rational, and not merely fortuitously convenient.

It should be clearly recognized that the only response treated in this paper is the growth of a test organ. For assay purposes, the injected substance has been regarded as a specific growth hormone. (Hence the use of 'substance' or 'hormone' as interchangeable terms.) The results should not be applied blindly to other types of assay. Thus, if an animal is injected with a thyrotropic substance, the response, measured by growth of the thyroid, is likely to be a logistic function of the dose. If, however, the response measured is, say, the rise in basal metabolic rate, there is no reason to expect a logistic relation. The resulting curve would have to be specifically investigated, both practically and theoretically for preference. In such cases, the obtaining of a sigmoid experimental curve is no guarantee that a logistic is the appropriate curve to fit. For example, considerations of probability or frequency may be involved, and integration of various types of frequency curve leads to sigmoid curves bearing a superficial resemblance to logistics.

In deriving the equations in this paper, a number of assumptions have been necessary. Here the aim has been to choose always the simplest assumption which does not conflict with known facts. Ignorance of facts may have led to unduly simple assumptions in some cases. One doubtful assumption is that the animal secretes the relevant hormone at a constant rate during the experimental period. This may be far from true, especially in the case of sex hormones, but the dosage of a hormone is usually massive compared with the animal's own output, and the latter may therefore be regarded as a small correction term for which a rough average value will suffice when, as is usual, a group of animals is treated.

It has further been assumed that a single hormone is involved. Natural growth of the test organ may, however, be due to a group of hormones, one of which is identical with the extrinsic hormone, or the substance administered may be a synthetic product chemically distinct from any natural hormone. In either of these cases the equations derived above will be somewhat in error as they stand, but may still be of the correct form. If it be permissible to assume that the various substances involved are independent in respect of secretion, excretion, and action on the test organ, then presumably the corrections would consist in replacing various single terms by sums of terms of the same type. In this case, equations which are linear in  $x$  or  $n$  would remain linear and give logistic dose-response or time-response curves as before, while equations of non-logistic final curves would become more complex, and would not become logistics. If the substances could not be treated as independent, some experimental data concerning their mutual effects would be needed for a comprehensive treatment, but here again the corrections would probably be unimportant except for very low dosage levels.

Another possible over-simplification is the assumption that the rate of absorption of hormone from a depot is proportional to its concentration in that depot. More strictly, the rate would be expected to vary as the difference in concentration between the depot and the body fluids.\* Here again the dosage is usually such that, initially at least, the concentration in the depot is immensely greater than that in the body fluids; hence the latter may be omitted without serious error. The error in the rate of absorption will usually only become appreciable when the depot is nearly exhausted. Unless the removal of hormone from the body fluids is extremely slow, the effect of the injection will also be nearly exhausted by this time, and an error which only becomes relatively large when the effect measured is absolutely very small will probably be undetectable in animal experiments. An attempt was made to treat the problem more rigorously by replacing equation (15) by

$$\frac{dA}{dt} = k_1 \left( \frac{x-A}{v} - \frac{z}{k} \right),$$

but the resulting system of equations proved intractable. A closer approximation to the solution than that actually employed could, no doubt, be obtained if this is considered worth while.

A rigorous treatment would also, presumably, involve some consideration of the partition of the hormone between the solvent and the body fluids. A solute tends to distribute itself between two solvents in such a way that, to a first approximation, when equilibrium is attained,  $c_1 = \text{constant} \times c_2^m$ ,  $c_1$  and  $c_2$  being the concentrations in the two solvents. The interpretation of the index  $m$  is that the solute molecules in solvent 2 are an  $m$ -fold polymer of those in solvent 1. It has been tacitly assumed above that the hormone is in the same state of molecular aggregation in the depot and body fluids (i.e.  $m = 1$ ).

A more general treatment of the material of this paper is not at present contemplated. It has been shown that the use of the logistic curve to represent dose-response relations has some theoretical justification. Some factors which may lead to deviations

\* If the dose were extremely high, the concentration of hormone in the body fluids might approach saturation, when a further correction would become necessary. This is perhaps most likely to occur in tablet implantations in small animals.

from this curve have been indicated. Further consideration of these factors may well be postponed until we have a larger body of data on which to judge the suitability of the simple logistic in practice.

Whether the logistic will attain the popularity of purely empirical curves, valid only over a limited range, will depend largely on the relative ease of fitting, and estimating the goodness of fit. It appears at present that methods of fitting logistics are somewhat tentative. A standard procedure, preferably not requiring the insertion of an experimental value of  $L$  (the limiting size of the organ), which may not be reached in some experiments, is very desirable. It is hoped that this problem will receive more attention from competent statisticians.

#### SUMMARY

1. Dose-response and time-response curves are derived on theoretical grounds for various types of assay in which the response measured is the growth of a test organ.

2. The basic assumption is that the growth of the test organ follows the well-known equation  $dy/dt = ay(L - y)$ ,  $y$  being the size of the organ at the time in question and  $L$  its maximum size. The coefficient  $a$ , however, is regarded not as a constant, but as a function of dose and time, the form of which depends on the conditions of the assay.

3. If the coefficient  $a$  is a linear function of the dose, the dose-response curve will be logistic both for a single dose and for a series of doses. The time-response curve will usually be more complex, but, as usually plotted, may tend to a simple logistic form under suitable conditions.

4. In the case of subcutaneous or intramuscular injections, it is shown (making simple assumptions regarding absorption and elimination of the dose) that

(a) for a single dose, the dose-response curve is a simple logistic, but not the time-response curve;

(b) for a series of subdoses, the dose-response curve is a logistic but not the 'smooth growth curve' (i.e. the time-response curve as usually plotted). The latter curve, however, tends to a simple logistic when absorption and elimination of each subdose are virtually complete by the time the next injection is given.

The above conclusions are less definite when the volume of the injectate depot varies appreciably than when it remains fairly constant during the period considered.

5. In the case of intravenous injection, a single dose will give a logistic dose-response curve, but a more complex time-response curve. Repeated doses will also give a logistic dose-response curve, while the 'smooth growth curve' will tend to a simple logistic form if elimination is sufficiently rapid.

6. If the dose were administered continuously at a uniform rate, the dose-response curve would be logistic, while the time-response curve would tend to this form if elimination were rapid, or if the time were long.

7. It follows from (6) that if the animal secretes the appropriate hormone at a uniform rate, the natural growth curve of the organ will not be strictly logistic, though it may be approximately so. If secretion and excretion have virtually reached equilibrium and body growth is slow compared with growth of the specific organ, the growth curve will be logistic. A logistic dose curve will also be obtained if the secretion of hormone varies with body growth in such a way that the concentration of hormone is constant.

8. When the dosage is by tablet implantation, the rate of absorption will presumably be fairly constant for a time, and the conclusions of (6) will apply. Over a longer period, the growth curve will not be logistic, neither will the relation of response to the original volume or area of the tablet be logistic.

9. Various factors which may qualify the above conclusions are discussed.

The author is greatly indebted to Dr J. O. Irwin, who has checked most of the mathematics in this paper and given very helpful criticism and advice on several points.

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Further references to the applications and fitting of logistics are given by Emmens [1940] and Pearl [1924].

# FERTILIZATION OF EGGS IN HYPOPHYSECTOMIZED RATS

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(Received 1 August 1945)

As the first step in an investigation of the hormonal requirements of the pregnant rat, we [Rowlands & Williams, 1943] examined the conditions under which ovulation can be produced after hypophysectomy. An injection of 40 i.u. of serum gonadotrophin (pregnant mares' serum) given 7-9 days after hypophysectomy brought the ovaries of immature or adult rats to a state where an injection of almost any gonadotrophic extract given 4-5 days later would produce ovulation. The next step has been to find out whether the eggs thus shed were fertilizable and sufficiently normal for their further development to be studied. The results of the examination are recorded below.

## MATERIAL AND METHODS

### *Rats*

Adult (100-120 g.) virgin females of the London Wistar strain bred in the Courtauld Institute were used.

### *Extracts*

An extract of pregnant mares' serum (PMS/IV) was prepared by the method of Rimington & Rowlands [1941] to the 'initial powder' stage. It contained 13 i.u./mg. Two preparations of chorionic gonadotrophin were used: CG/R, a commercial preparation containing 113 i.u./mg. and UP/31, machine-scrapings of the International Standard, containing 10 i.u./mg.

### *Injections*

All rats received a subcutaneous injection of 40 i.u. of serum gonadotrophin 6-9 days after hypophysectomy and 4 days later a subcutaneous injection of 50 i.u. of chorionic gonadotrophin. The injections were arranged so that ovulation, which occurs 13-14 hr. after the injection of the chorionic gonadotrophin, should occur about midnight.

### *Mating*

Attempts at artificial insemination, using the technique of Blandau & Jordan [1941], were unsuccessful so the rats were mated normally. Pairs of female rats were placed in cages with four males immediately after the chorionic gonadotrophin had been injected. They were separated the next morning. Mating was indicated by the presence of a vaginal plug or of sperm in the vaginal smear. The rats which did not mate after the first course of injections were given a similar course starting 14-21 days after hypophysectomy.

*Inspection of eggs*

The rats were killed on the second day after mating had been established by vaginal examination. The Fallopian tubes were trisected under low-power magnification [Rowlands, 1942]; the contents of each segment were expressed separately and examined microscopically.

*Controls*

Some normal virgin females of the same weight were killed two days after mating had been established by vaginal examination. The tubal inclusions were examined as described above.

## RESULTS

*Mating*

In all 116 hypophysectomized females were placed with intact males and 42 of them (36 %) mated. The incidence of mating was the same following the first and second course of injections. The cause of this low incidence and the factors governing the reception of the male by the hypophysectomized female rats are being separately investigated.

*Ovulation*

Of 50 hypophysectomized rats that mated or were examined, 32 had ovulated. This proportion of 64 % ovulation is not significantly different from that of 80 % among the 10 rats of similar size we injected with the same amounts of the two hormones in 1943. When the interval between operation and first injection, or between the start of the two courses of injection, was longer than 8 days there was some decline in the proportion of rats ovulating (33 %), but as there were only 6 rats in the group the difference is not significant. Otherwise the incidence of ovulation and the number of eggs shed in the rats that did ovulate are remarkably constant, as shown by the results analysed in Table 1. There is no difference between the effects of the first and second courses of injection. In the control series all the 23 rats that mated had ovulated.

Table 1. *Incidence of ovulation in experimental rats*

Course of injections	Interval* (days)	No. of rats	Rats ovulating		No. of ova per ovulation
			No.	%	
1st	6	11	8	73	13.6
1st	7	15	10	67	10.3
1st	6-9	32	21	66	12.0
2nd	7-12	18	11	61	12.0
1st and 2nd	—	50	32	64	12.0

\* Interval between operation and 1st serum gonadotrophin injection (1st course) or between 1st and 2nd injections of serum gonadotrophin.

*Fertilization*

The eggs expressed from the Fallopian tubes of the control and experimental rats are classified in Table 2 according to their stage of cell division.

All the eggs from the control rats had segmented but 21 (9 %) of those from the experimental rats were unicellular. The occurrence of fertilization in the unsegmented eggs was not determined. If these eggs are fertilized then it is probable

that either their rate of cleavage is retarded or that they are incapable of segmentation.

Table 2. *Number of eggs and stages of cell division in mated normal and hypophysectomized rats*

	Normal rats		Hypophysectomized rats	
No. of rats	13		23	
Total no. of eggs	64		239	
No. of eggs per rat	4.9		10.4	
No. of normal eggs per rat	4.7		7.1	
Stage of cell division	Normal rats		Hypophysectomized rats	
	No.	% of total	No.	% of total
1-cell	0	0	21	9
2-cell	28	44	127	53
3-cell	4	6	7	3
4-cell	29	45	9	4
Degenerate (fragmented)	3	5	75	31

About one-third (31 %) of the eggs recovered from the hypophysectomized rats were degenerate. These eggs were fragmented and consisted usually of one normal-sized blastomere of somewhat irregular shape with an eccentrically situated nucleus and a varying number of small enucleate bodies of different sizes. They resemble very closely the unfertilized fragmented eggs of mice described and illustrated by Lewis & Wright [1935]. If the degenerate eggs in our series have been fertilized then their development has proceeded abnormally beyond the two-cell stage. The proportion of such eggs in the experimental rats is significantly greater than in the controls. The figures given in Table 3 show that this high incidence of degeneration is not related to the number of eggs discharged from each ovary or to the course of injection involved.

Table 3. *Analysis of incidence of degeneration among eggs from experimental rats*

Course of injection	No. of eggs per tube	No. of tubes	No. of eggs	Degenerate eggs	
				No.	%
1st and 2nd	1-5	27	74	23	31
1st and 2nd	6-10	11	77	21	27
1st and 2nd	11-15	5	59	15	25
1st	1-15	34	167	43	26
2nd	1-15	9	43	15	35

The remaining eggs (60 %) in the experimental rats were microscopically normal. Dr E. C. Amoroso kindly examined 39 representative two-cell eggs from the experimental rats and reported them to be normal, fertilized eggs. The only abnormality in these eggs appears to lie in their rate of segmentation, since only 4 % of all the eggs in the hypophysectomized rats were in the four-cell stage as compared with 45 % among the eggs from the normal rats. This observation is discussed further below.



## COMMENT

*Fertility of eggs*

The fact that 30 % of the eggs shed in the experimental rats were degenerate and a further 9 % may be infertile, is not related to the high number of eggs shed in these rats. If it were, then the proportion of degenerate and one-cell eggs should increase parallel with the number of eggs per tube, and this does not happen. The high incidence of abnormal eggs in the experimental rats may be caused by ovulation having occurred prematurely or abnormally late in relation to the age of the ovum. When we determined the conditions necessary to produce ovulation in hypophysectomized rats we chiefly used immature rats and it may be that slight modifications of the doses are needed for the adult rat. In any case the previous experiments were quantitative only, and the fecundity of the eggs was not considered. Another uncontrolled factor is the relation between the times of copulation and ovulation which, if not optimal, will result in decreased fertility [Soderwall & Blandau, 1941].

*Rates of cleavage and tubal passage*

Gilehrst & Pincus [1932] examined the eggs from the Fallopian tubes of a small number of rats after mating. They found that the first cleavage took place 27-42 hr. after copulation and no two-cell eggs were found later than 58 hr. after copulation. As no eggs were found earlier than 8 hr. after copulation it is assumed that ovulation occurred at about this time after mating.

The eggs from our experimental rats were examined 58-66 hr. after ovulation; the presence of one-cell eggs and the low proportion of three- and four-cell eggs among them suggest an abnormally slow rate of cleavage. On the other hand, if the one-cell eggs are not fertilized and we add the fragmented eggs to the four-cell eggs on the assumption that their cleavage has proceeded abnormally only after the two-cell stage then the differences in rate of cleavage are no bigger than might occur in different rat colonies in different countries.

Our own control series does not provide any useful information on this point as the female rats were smeared only once daily, so that ovulation can only be timed within 24 hr. limits.

The same qualifications prevent any great significance being attached to our impression that the eggs of the experimental rats were higher in the Fallopian tubes than they were in the control rats.

*General*

Further investigations are being carried out in the hypophysectomized rat in an effort to increase the proportion of normal eggs and to determine whether there is, in fact, any delay in the rate of segmentation or tubal passage. At the same time we feel that the number of apparently normal eggs is high enough to warrant examination of their further development.

## SUMMARY

1. Ovulation was produced in hypophysectomized female rats by injections of serum and chorionic gonadotrophins. These rats were mated with normal males and their eggs collected 2 days later and compared with eggs from normal mated females.

2. The average number of eggs recovered from the Fallopian tubes of the experimental rats was 10.4; that from the normal rats was 4.9.

3. In the experimental rats 60 % of the eggs were normal fertilized two-cell, three-cell, or four-cell eggs; 9 % were one-cell eggs of uncertain normality, and 31 % were degenerate. There were only 5 % of degenerate eggs in the normal rats.

4. The proportion of degenerate eggs is not related to the number of eggs shed per ovary.

We are very grateful to the Council of the Middlesex Hospital Medical School for providing P.C.W. with laboratory facilities and a personal grant during the course of this investigation; to Dr E. C. Amoroso for examining some of the eggs; to Dr F. X. Aylward for a supply of PMS/IV; and to Mr I. A. Hepple for technical assistance.

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# FURTHER OBSERVATIONS ON THE FORMATION OF 'GHOSTS' IN SUBCUTANEOUSLY IMPLANTED TABLETS

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(Received 22 September 1945)

The formation of a protein deposit in subcutaneously implanted tablets of hexoestrol and other oestrogens was first noted by Folley [1942], who also found [1944] that a similar reaction occurred when tablets were incubated *in vitro* with serum, plasma, or solutions of proteins. This deposit is revealed when the tablet is removed and the residual active substance dissolved away in alcohol or ether. Usually the deposit, thus exposed, takes the form of a coherent, semi-transparent or opaque body of the same shape, and often approximately of the same size, as the original tablet—hence the term ghost, by analogy with the stroma of the red blood corpuscle after haemolysis. The fact that the ghost has structure and coherence shows that it is not composed merely of the dry matter of the body fluids present in the tablet when it is removed. On the contrary, it is undoubtedly laid down in the interstices of the tablet as the result of some organized process. The larger the interstices the more substantial the ghost, and vice versa. Thus, when a tablet composed of diethylstilboestrol and lactose is implanted the lactose dissolves away very rapidly leaving a highly porous structure which soon acquires a very substantial ghost. Conversely, when a fused block containing no interstices is implanted, the ghost takes the form only of a hollow peripheral capsule, which is quite distinct from the capsule of connective tissue growing round the block or tablet in the course of a few weeks [Deanesly & Parkes, 1943; Cowie & Folley, 1945].

The coherence of the ghost suggests that some physical or chemical change takes place in the protein laid down in the tablet. Folley [1944] has suggested that the ghost ultimately consists of scleroprotein. It is possible, therefore, that the formation of a ghost might depend on the chemical nature of the substance of which the tablet is composed. Ghost formation has been reported in tablets of nearly all the steroid hormones, including oestrone, oestradiol, testosterone, progesterone, deoxycorticosterone acetate, and ethisterone [Deanesly & Parkes, 1943; Bishop & Folley, 1943] as well as in those of the synthetic oestrogens. No systematic examination, however, has been made of the occurrence of ghosts in tablets of esters and other derivatives of these substances, or in those of substances of other types. A large series of compounds were therefore examined by the following technique.

## TECHNIQUE

Tablets of 10, 25, 50 or 75 mg., according to the amount of material available, were prepared in a hand tablet-making machine. The pressure used was kept as constant as possible, since this, together with the physical state of the material, affects the

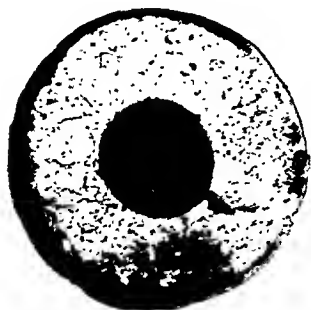


FIG. 1. Androstenedione. Original weight  
50 mg.  $\times 13$ .

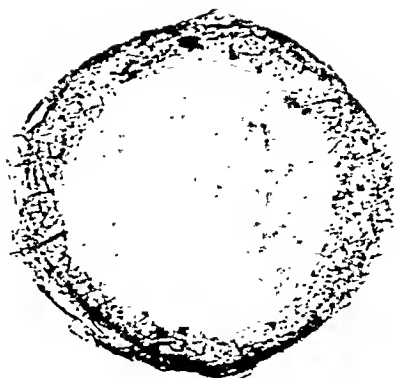


FIG. 2. Oestrone methyl ether. Original weight  
25 mg.  $\times 16$ .

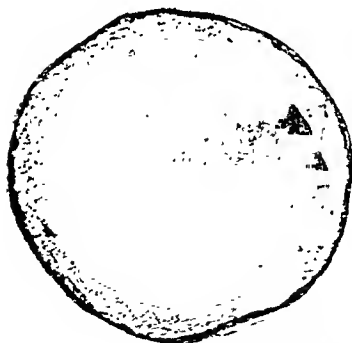


FIG. 3. Lumisterol. Original weight  
50 mg.  $\times 12$ .

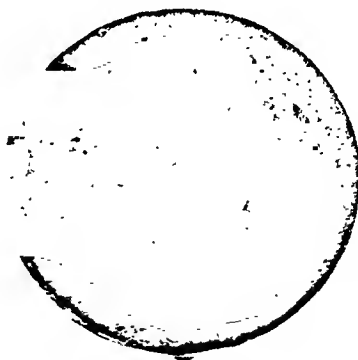


FIG. 4. Benzyl sulphanilamide. Original  
weight 75 mg.  $\times 11$ .

Tablets implanted for 5 days and partially dissolved in alcohol, showing residual tablet in the centre surrounded by halo of ghost.

The photographs are the work of Mr F. V. Welch.



size of the interstices in the tablet. Two or more tablets of each substance were implanted subcutaneously into rats for 5 days, while other similar tablets were incubated in ox plasma for 3 days at 37°C. On removal the tablets were washed and extracted with a suitable solvent, usually alcohol or ether, to remove the residual original substance. The resulting ghosts were examined under a hand lens or a low power binocular, and in many cases sectioned histologically as described by Deanesly & Parkes (1943). Photographs were taken of certain of the ghosts cleared in xylol.

The ghosts from the *in vivo* experiments were classified according to their density, as follows:

- ++++ substantial ghost opaque macroscopically and with thick meshwork histologically;
- +++ ghost still semi-opaque, but meshwork less heavy;
- ++ ghost semi-transparent macroscopically, and meshwork thin and irregular histologically;
- + ghost little more than a transparent envelope;
- 0 no coherent residue.

For the *in vitro* experiments the grading was as follows:

- ++++ white coherent solid ghost;
- +++ opaque solid ghost—shrunk;
- ++ semi-transparent membrane;
- + pieces of membrane;
- 0 no coherent residue.

The results obtained with different tablets of the same substance showed good agreement, and the *in vitro* experiments gave substantially the same results as those carried out *in vivo*.

## RESULTS

All the substances examined produced ghosts to some degree in one or both series of experiments. Free oestrone, oestradiol, testosterone, androsterone, as well as free diethylstilboestrol and hexoestrol and several of the other synthetic substances all produced very substantial ghosts. The esters, on the other hand, produced, generally speaking, comparatively flimsy ghosts which were manipulated with some difficulty. It is difficult, however, to interpret this finding in terms of chemical structure, since oestrone methyl ether, diethylstilboestrol dimethyl ether, and androstenedione produced good ghosts. The inert steroids, cholesterol, lumisterol, and sitosterol gave rather thin ghosts. Tablets of cholesterol and dextrose, containing residual dextrose after implantation, did not give a coherent ghost, presumably because the deposition of protein was interfered with by the presence of dextrose in the interstices of the cholesterol. Tablets of benzyl sulphanilamide with dextrose containing no residual dextrose after implantation gave, on the other hand, very substantial ghosts similar to those already recorded [Deanesly & Parkes, 1943] in tablets originally consisting of diethylstilboestrol with lactose.

In addition to the substances listed in Table 1, experiments were carried out with several other substances, but were unsuccessful for one reason or another. Tablets of oestriol triacetate, silica, and sulphanilamide disappeared during the course of the 5 days' implantation; tablets of ethisterone, stilboestrol dibenzoate, and oestradiol

dibenzoate were almost insoluble in the usual organic solvents at room temperature and prolonged refluxing was thought likely to be incompatible with the survival of a ghost. Animals receiving tablets of adrenaline and insulin died within a day, so that no results were obtained with these substances.

## SUMMARY

The formation of ghosts has been examined in tablets of 67 different substances. The phenomenon is not restricted to the hormonal steroids and biologically related substances. It occurs in compressed tablets of a wide range of synthetic substances, including benzyl sulphanilamide, and in all the inert steroids examined.

Table 1. *Ghost formation in tablets of various substances*

Substance	Ghost formation	
	<i>In vivo</i>	<i>In vitro</i>
Oestrone	++++	++++
„ iso-butyrate	++	++
„ laurate	++	—
„ palmitate	+	+
„ methyl ether	++++	—
Oestriol triacetate	—	+
Oestradiol	++++	+++
„ dipropionate	+++	++
„ monobenzoate	+++	—
„ 3-benzoate 17- <i>n</i> -butyrate	++	+
„ dibenzoate	—	++
Ethinyl oestradiol	+++	+
Diethylstilboestrol	++++	++++
„ diacetate	++	++
„ dipropionate	++	++
„ di- <i>n</i> -butyrate	+	+
„ di-iso-butyrate	+	+
„ diacetate	++	+
„ diacrylate	+	+
„ dilaurate	++	+
„ dipalmitate	+	+
„ dimethyl ether	+++	++
„ ditrimethyl acetate	+	+
Hexaestrol	++++	++++
Dienestrol	++++	++++
Tosterone	++++	++++
„ 3-acetate 17-propionate	+	+
„ 3-acetate 17- <i>n</i> -butyrate	++	++++
„ propionate	++	+++
„ 3:17-dipropionate	++	++++
„ propionate oxime	+	++
„ benzoate	++	++
„ hydroxylamino acetate	—	+
Methyl testosterone	+++	+++
Androsterone	++++	++++
<i>trans</i> -Dihydroandrosterone	++	++
Androstenodione	+++	—
Androstene-3:17- <i>trans</i> -diol	+	+
<i>trans</i> -Androstenediol 17-benzoate	+	++
<i>cis</i> -Androstenediol 3-benzoate	++++	++
Androstane 3- <i>cis</i> -17- <i>trans</i> -diol	++	+++

Table 1 (*cont.*)

Substance	Ghost formation	
	<i>In vivo</i>	<i>In vitro</i>
H 1, $\beta$ 2'-Diamino- $\alpha\beta$ -dinaphthyl	++	++
H 2, 1:2'-Azonaphthalene	—	+
H 3, 9-Methyl-1:2-benzfluorene	++	+
H 5, 9-Methyl-3:4-benzfluorene	+	+
H 6, Triphenyl iodo-ethylene	+++	—
H 7, Triphenyl bromo-ethylene	++	++
4:4'-Dihydroxydiphenyl	—	++++
4-Hydroxypropio-phenone pinacol	++++	++++
$\alpha$ -(4-Hydroxyphenyl) stilbene	++++	+++
$\alpha$ -Ethyl triphenyl ethylene	++	+++
$\alpha$ -Ethyl- $\beta$ ( <i>p</i> -hydroxyphenyl) stilbene	++++	+++
Dihydroxy-di- $\alpha$ -naphthyl-acenaphthene	++	—
4-Hydroxydiphenyl	++	++
4-Hydroxystilbene	+++	+++
Triphenyl chloroethylene	++	++
Triphenyl ethylene	+	+
Triphenyl carbinol	—	++
4- <i>tert</i> -Amyl phenol	—	+
Benzyl sulphanilamide	+++	—
Capryl sulphanilamide	++	—
Sulphathiazole	+++	—
Sulphapyridine	++	—
Cholesterol	++	—
Lumisterol	++	—
Sitosterol	++	—

Our thanks are due to Prof. E. C. Dodds for most of the synthetic oestrogens, to Dr A. Haddow for the H series of compounds, and to Glaxo Laboratories, British Drug Houses Ltd., and Ciba Ltd. for various other substances. The tablets were prepared by Mr F. Crisp.

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# RELATION BETWEEN IODINE CONTENT AND BIOLOGICAL ACTIVITY OF THYROID PREPARATIONS

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(Received 22 September 1945)

A recent study of artificially iodinated proteins showed that biological activity, in relation to acid-insoluble iodine content, was very low compared with that of dried thyroid gland [Deanesly & Parkes, 1945*b*]. Since the main active principle (thyroxine) is the same in both types of preparation [Ludwig & von Mutzenbecher, 1939] it was concluded that the artificially iodinated proteins at present available contain relatively large amounts of iodine-containing substances which are acid-insoluble but have little or no biological activity. In the case of thyroid preparations it is usually supposed that all the acid-insoluble iodine is thyroxine-iodine and dried thyroid powder is assayed by the chemical estimation of acid-insoluble iodine. Even in thyroid powders, however, some investigators have not found a very close relation between acid-insoluble iodine and biological activity. Thus, Gaddum & Hetherington [1931] concluded that the acid-insoluble iodine content of dried thyroid preparations did not give a reliable indication of capacity to raise the carbon dioxide output of mice, and Wokes [1938] and Dutt & Mukerji [1942] found that the biological activity, as tested on tadpoles, agreed better with the total iodine content than with the acid-insoluble iodine content. The biological methods used by these authors, however, were not very quantitative, and the elaboration of what promises to be a more generally useful technique [Deanesly & Parkes, 1945*a*] suggested that a reinvestigation of thyroid powders would be useful. With a view to securing differences in biological activity, ten preparations were made from the thyroid glands of five different species of animal—ox, sheep, pig, horse, and dog. The present paper records the biological activity of these preparations in relation to their total and acid-insoluble iodine contents.

## TECHNIQUE

### *Preparation of glands*

Fresh thyroid glands of ox, sheep, horse, and pig were obtained from various London slaughter-houses, the tissue being kept frozen during collection and transit, and until extraction or dehydration. One batch of glands from each of these species was prepared by each of two methods. The first method consisted of desiccating the tissue in acetone followed by ether. The dried tissue was then ground as finely as possible in a coffee mill. Much of the resulting powder was still too coarse for assay by the tadpole technique or for reasonable certainty of homogeneity. As there seemed little chance of reducing it all to an adequately fine powder with the apparatus available, the material was passed through two sieves, 30 mesh and 60 mesh, giving three fractions (*a*) passed 60, (*b*) passed 30 but not 60, (*c*) failed to pass 30. The relative amount falling into these three fractions varied considerably with the species

of animal and with the degree of grinding. This point, however, is immaterial since there was no intention of trying to assess the total activity of the various glands. Only the finest fraction (labelled 'a') was assayed. A list of these preparations is given in Table 1.

Table 1. *Preparations of dried thyroid gland*

Species	Batch no.	Approximate weight (g.) of		
		Wet gland	Dried gland	Fraction 'a'
Ox	1	306	72	4
Sheep	1	458	92	16
Horse	1	609	13	7
Pig	1	282	76	39
Dog	1	92	21	7
Dog	2	64	15	4

The second method consisted of the preparation of crude thyroglobulin by a simplification of the technique described by Harington & Salter [1930]. The glands were trimmed, minced finely, weighed, and shaken in an equal volume of a solvent composed of 1% NaCl and 0.02% NaOH. After 3 hr. the residue was removed by filtering through muslin and reshaken for 3 hr. with fresh solvent. The two extracts were combined, chilled, skimmed for fat, adjusted to pH 5.0 and precipitated with 4 vol. of acetone. The precipitate was collected and dried with fresh acetone and ether. A list of these preparations is given in Table 2. It will be seen that the percentage yield did not vary much from species to species.

Table 2. *Preparations of crude thyroglobulin*

Species	Batch no.	Approximate weight (g.) of		Extract as % wet tissue
		Wet tissue	Crude extract	
Ox	2	606	97	16
Sheep	3	722	104	14
Horse	3	335	42	13
Pig	2	574	93	16

The collection of dog thyroids was more difficult, and they were put into acetone on removal from the animal. Only the first method of preparation could therefore be applied to this material.

#### *Biological assay*

The preparations were assayed on *Xenopus* tadpoles by the method described by Deanesly & Parkes [1945*a*]. After preliminary trials to discover the dosage required to give a suitable response, a series of tests was carried out. The commercial thyroid preparation 628423 which had previously been the subject of co-operative iodine estimations was used as a standard reference substance, and assigned an activity of 1.0 in each test. This preparation had a total iodine content of 0.27% and an acid-insoluble iodine content of 0.10%.

The results of each test were obtained by plotting log dose against probit response, as previously described [Deanesly & Parkes, 1945*a*; see also Miller & Tainter, 1944]. From the graphical representation of each test, the slope (*b*) of the standard line was calculated from the formula

$$b = \frac{2}{\log (\text{dose probit } 6) - \log (\text{dose probit } 4)}$$

An approximate weight was then calculated for each test of each substance, using the formula

$$W = \frac{1}{\sigma^2} = \frac{1}{\frac{1}{b^2} \left\{ \frac{2}{N_{st}} + \frac{2}{N_t} \right\}},$$

where  $N_{st}$  = total number of tadpoles on standard, and  $N_t$  = total number of tadpoles on test substance.

Successive tests were carried out until the total weight of the tests for each substance had a sum of approximately 1000. The weighted mean potency of each preparation in relation to the standard was then calculated from the formula

$$\bar{X} = \frac{\sum W X}{\sum W},$$

where  $\bar{X}$  is the weighted mean log activity and  $X$  is the log activity.

Finally, the limits of error for  $P=0.95$  were determined from  $\bar{X} \pm 2\sigma$ , where  $1/\sigma^2 = \sum W$ .

Between four and eight satisfactory tests of each substance were required (see Table 3). Unsatisfactory tests were mainly due to unpredictable variation in the sensitivity of different batches of tadpoles, resulting in failure to obtain two suitable response levels for a substance.

#### *Iodine analyses*

These were carried out in duplicate by the *British Pharmacopoeia*, 1932 (indirect) and the *British Pharmacopoeia* 1936 (direct) methods. Agreement was reasonably good. In cases of discrepancy the figures given by the indirect method were used. Throughout the paper the term 'acid-insoluble iodine content' refers to the acid-insoluble iodine determined after hydrolysis.

#### RESULTS

The biological activities and iodine contents of the various preparations are given in Table 3. It will be seen that all except the two dog thyroid preparations were more active than the standard. This is not unexpected since the latter had been diluted with lactose to conform with *British Pharmacopoeia* iodine standards. Dog 1a was markedly lower in activity (0.42) than any other preparation, and the difference was highly significant. Dog 2a was much better (0.95) but was significantly less active than the other preparations, except sheep 1a which had an activity 1.2

Table 3. *Analyses and assays of thyroid preparations*

Preparation	Iodine content (%)		No. of assays	Weighted mean potency	Limits for $P=0.95$
	Total	Acid-insoluble			
Dog 1a	0.25	0.07	7	0.42	0.35-0.49
Dog 2a	0.36	0.08	7	0.95	0.81-1.11
Sheep 1a	0.49	0.11	7	1.20	1.03-1.39
Sheep 3	0.44	0.09	7	1.38	1.22-1.56
Ox 1a	0.61	0.11	4	1.38	1.21-1.59
Ox 2	0.68	0.11	8	1.43	1.24-1.65
Horse 3	0.49	0.10	8	1.45	1.28-1.65
Pig 1a	0.66	0.14	7	1.65	1.45-1.87
Horse 1a	0.57	0.14	8	1.72	1.52-1.94
Pig 2	0.59	0.11	6	1.86	1.60-2.17

that of the standard. Ox 1*a*, ox 2, sheep 3, and horse 3 all had very similar activity about 1.4 that of the standard. The remaining three preparations, two of pig and one of horse thyroids, had greater activity, the highest being 1.86 that of the standard. The last preparation was significantly more active than any of the medium preparations in the 1.4 range. Considering only the preparations of crude thyroglobulin, and taking into account the yield obtained and the biological activity shown, it would seem that pig thyroids contain more active material per gram than those of ox, sheep, or horse.

The acid-insoluble iodine figures for the ten preparations range from 0.07 to 0.14 %, the dog material giving the poorest values, and pig and horse the best.

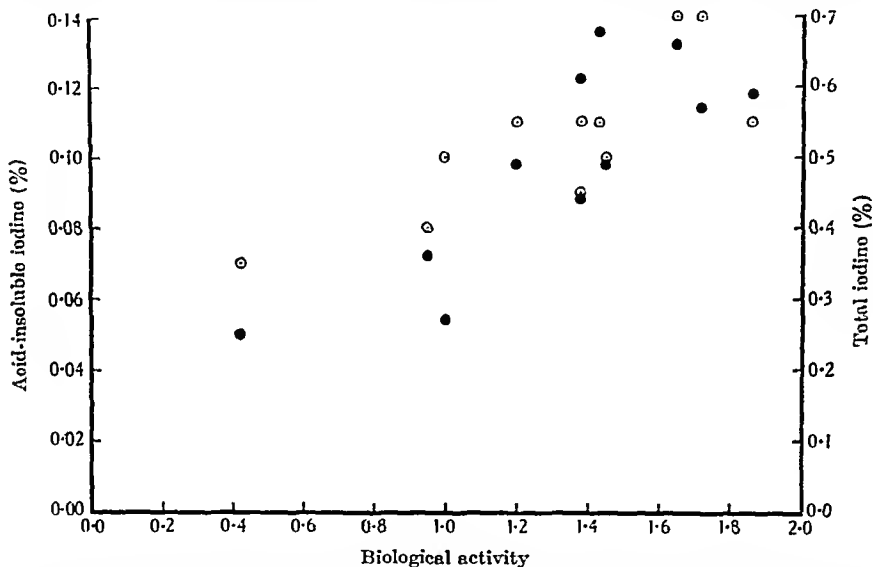


FIG. 1. Relation between biological activity and (a) total iodine, (b) acid-insoluble iodine, in eleven preparations of thyroid gland from five different species. Biological activity is expressed in terms of that of a standard preparation (628423) whose activity is given the value 1.0. ● = total iodine; ○ = acid-insoluble iodine.

Considering all the preparations, the range of acid-insoluble iodine values is from 1 to 2, while the range of biological activity is from 1 to 4. Omitting dog 1*a*, however, the range of both is roughly the same, which is what would be expected if the acid-insoluble iodine were all thyroxine iodine.

The detailed relation between acid-insoluble iodine and biological activity is shown in Fig. 1, which includes a point for the standard preparation. It will be seen that, allowing for the combined error of the chemical and biological assays, the values for all the preparations except dog 1*a* are compatible with a linear relationship between acid-insoluble iodine and biological activity, and with the line relating the two passing through the origin. This would imply that the acid-insoluble iodine in thyroid preparations is all thyroxine iodine, or else that the latter forms a constant proportion of the whole acid-insoluble iodine. The data for dog 1*a* are not quite concordant; the preparation has less biological activity than would be implied by its

acid-insoluble iodine content. This single anomaly, however, cannot carry much weight. It is of some interest to note that even if the acid-insoluble iodine of dried thyroid is all thyroxine iodine, the thyroxine content of preparation 628423 will be only about 0.15%, i.e. about 1/700. Yet, in the same type of biological test, *l*-thyroxine is only about 100 times as active as this preparation. The discrepancy, which has complicated much discussion about iodine content and biological activity, is presumably due to the greater activity of thyroxine in protein combination; it cannot be due, in more than a negligible degree, to the presence of di-iodotyrosine in thyroid preparations.

Table 3 shows that there is a fairly close relation between acid-insoluble iodine and total iodine, and it may be taken that the former represents a fairly constant proportion of the latter. Both values therefore give very similar pictures when plotted against biological activity (Fig. 1). No doubt this fact accounts for the doubt expressed in the literature whether total iodine or acid-insoluble iodine gives a better indication of biological activity. On the basis of the results given above, both are about equally good, though the comparative lack of preparations of low iodine content has prevented the presentation of a completely convincing range of data. It should be emphasized, however, that the essential relation of biological activity is with acid-insoluble iodine, that with total iodine being more or less fortuitous.

#### SUMMARY

1. Ten preparations of thyroid glands from five different species of animals have been examined for biological activity by the *Xenopus* tadpole test.

2. There was a fairly close relation between total iodine and acid-insoluble iodine in these preparations, and the biological activity agreed about equally well with both figures.

3. The data are concordant, apart from one anomalous preparation, with the supposition that thyroxine iodine forms a fairly constant proportion both of the acid-insoluble iodine and of the total iodine.

4. No positive evidence was obtained of the presence in these thyroid preparations of inactive substances containing acid-insoluble iodine, and it seems likely that in accordance with current opinion the acid-insoluble iodine of thyroid preparations, unlike that of artificially iodinated proteins, is all thyroxine iodine.

I am much indebted to Dr E. C. Amoroso who arranged the collection of dog thyroids, and to Mrs M. Dix of Boots Pure Drug Co. and Miss S. Carswell for statistical assistance. The iodine analyses were carried out by Mr R. G. Steele, I.C.I. (Explosives) Ltd., to whom my best thanks are due. The standard thyroid powder was kindly supplied by Boots Pure Drug Co.

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# GONAD STIMULATION BY ANDROGENS IN HYPOPHYSECTOMIZED PIGEONS

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(Received 4 October 1945)

There have been many contradictory reports of the effects of androgens on the testes. In the rat the most frequently noted effect has been an adverse one (see review by Moore [1939]), and in the pigeon, too, atrophy of the testes has been produced by testosterone injections [Chu, 1940]. This adverse effect is presumably due to an inhibition of gonadotrophin production or secretion.

On the other hand, the testis may be stimulated in immature or adult, intact or hypophysectomized, ground squirrels [Wells & Moore, 1936; Wells & Gomez, 1937] or in immature rats [Shay, Gershon-Cohen, Paschkis & Fels, 1941] by androgen injections. In hypophysectomized animals spermatogenic activity may be maintained by injections of androgen [Cutuly & Cutuly, 1940; Chu, 1940; and earlier reports by others] or when the testes have atrophied spermatogenesis may be partially restored in the same manner [Selye & Friedman, 1941]. The present report describes similar results in male pigeons and ovarian stimulation following androgen injection in female pigeons.

## MATERIAL AND METHODS

Pigeons obtained from a local dealer were kept in cages (ten per cage) and given wheat and corn twice daily with occasional vegetables. The birds were sexed by laparotomy and hypophysectomy was performed trans-buccally [Chu, 1940]. The birds were not injected until at least 30 days after operation, and as many died meanwhile few were available for investigation.

In the male birds the left testis was removed before the injections were started to serve as a control to the right testis which was removed at the end of the experiment. The testes were weighed after fixation and transferred to 70 % alcohol.

In the females ovarian conditions were determined by examination at laparotomy.

## RESULTS

### *Effects of testosterone on the atrophic testis*

Testosterone propionate was injected daily (2 mg. in 0.5 ml. of sesame oil) into eight birds for 7-21 days starting 30 days after hypophysectomy. The right testis was removed on the day after the last injection and compared with the left testis which had been removed on the day preceding the first injection. Four birds survived the full course of twenty-one injections, the others dying after seven, eight and twelve injections. Three hypophysectomized birds had their testes removed at similar times though receiving no injections.

The results given in Table 1 show that there was no significant difference between the weights of the right and left testes of the control birds, while in all cases but one

the right testis was heavier than the left one in the injected birds, particularly in those surviving the full 3 weeks. The exceptional bird (P39) in which the right testis did not differ from the left was found to be incompletely hypophysectomized.

Table 1. *Effect of testosterone (2 mg. daily) on the atrophic testes of hypophysectomized pigeons*

Bird no.	Left testis			Injection period days	Right testis		
	Days after hypophysectomy	Weight mg.	Tubular diameter $\mu$		Days after hypophysectomy	Weight mg.	Tubular diameter $\mu$
Experimental birds							
P44	31	20.4	40-60	20	51	69.6	80-120
P79	32	33.5	90-120	21	53	370.7	150-230
P98	31	44.5	40-60	21	52	150.2	60-180
P39	56	12.1	40-60	21	77	15.0	35-55
P43	31	26.1	45-60	12	43	78.5	95-115
P56	34	13.2	40-60	8*	42	32.0	75-90
P87	30	8.8	60-70	12	42	33.0	80-120
P55	33	24.1	60-80	7	40	41.7	80-120
Control birds							
P82	35	21.8	40-60	—	56	32.0	—
P113	30	48.0	40-60	—	51	34.5	—
P283	35	96.0	—	—	56	44.0	—

\* Daily injection of 3 mg. of testosterone.

Histologically the left testes removed before injection showed extreme degeneration with decreased tubular size and an increase in intertubular connective tissue. The Leydig cells were scattered and shrunken with pyknotic nuclei. The germinal epithelium had few mitoses and spermatogenesis was arrested at the primary spermatocyte stage. The right testes of the control birds removed 3 weeks later were similar.

The right testes of the birds injected during 21 days had enlarged tubules, and a reduction of the intertubular connective tissue with active spermatogenesis and mature sperms in the majority of tubules. The histological picture was quite normal except that the Leydig cells were still degenerate. The incompletely hypophysectomized bird showed no restoration of function and those injected for 7, 8 and 12 days only partial restoration in that spermatogenesis did not proceed beyond the secondary spermatocyte stage.

#### *Effects of testosterone and oestrone on the atrophic ovary*

Only three hypophysectomized birds survived more than 1½ years. In each case laparotomy disclosed an atrophic ovary with no follicles more than 1 mm. in diameter before the injections were given.

One bird was injected intramuscularly with 2 mg. of horse pituitary extract (AP118B) in aqueous solution daily for a fortnight. On the day following the last injection many yolk-laden follicles were present in the ovary, the largest being 1 cm. in diameter. The response to pituitary gonadotrophin was obviously normal.

The two remaining birds received a course of oestrone injections in one case preceded and in the other followed by a course of testosterone injections. The two courses of treatment were separated by about a month and each consisted of twenty daily injections of 4 mg. of the substance concerned. At the end of the oestrone injections the ovaries contained only very small follicles. After the testosterone injections the ovaries were markedly stimulated, having many yolk-laden follicles (the largest 4-6 mm. in diameter), showing clearly that testosterone may act directly on the ovary in the absence of the pituitary gland.

#### *Effects of androgens in intact pigeons*

Since androgens cause testicular atrophy in normal pigeons and stimulation in hypophysectomized pigeons, it is possible that pituitary gonadotrophin may antagonize androgens. If this is so then the testes of animals in the non-breeding season or of immature animals would be stimulated by androgen since gonadotrophin liberation is minimal at such periods.

Six mature male pigeons were injected with androgen during November and December 1941 while four similar untreated birds served as controls. Two of the injected birds received 3 mg. of testosterone propionate daily for 20 days and four received the same daily dose of androsterone for 13-20 days.

The average weight of the testes in the control birds was 78 mg. and in the experimental ones 45 mg. There was no spermatogenesis in progress in any bird so that it may be concluded that sexually quiescent pigeons are not stimulated by these doses of androgens.

Four immature male pigeons who had just assumed their permanent plumage had their left testes removed. Two were then given 4 mg. of testosterone propionate daily for 20 days while the other two served as controls. No testicular stimulation was produced. Four immature female pigeons were similarly treated and in this case it was found that androgens exerted a considerable stimulating effect on the ovary.

#### DISCUSSION

The production of full spermatogenesis in the atrophic testes of hypophysectomized pigeons confirms previous findings in mammals and indicates that androgen has a gonadotrophic action in the male. The fact that this action does not appear in the presence of pituitary tissue, when the gonadal functions may be actually suppressed by such large doses, suggests an antagonism between androgen and pituitary gonadotrophin. This is supported by the finding that androgen, even in large doses, does not stimulate spermatogenesis in immature pigeons or in adults during the non-breeding season.

This gonadotrophic action of androgen suggests that it may be possible to maintain spermatogenesis in hypophysectomized animals with luteinizing hormone, which is known to stimulate the Leydig cells. Preliminary experiments in which our two crude pituitary extracts (AP118B and AP81B) were treated with picric acid to inactivate the follicle-stimulating activity [Fevold, 1939], or with trypsin to destroy the luteinizing activity [McShan & Meyer, 1938], show that both were capable of partially restoring the spermatogenic activity of the atrophic testes in hypophysectomized pigeons, and there was some suggestion that the extracts treated with picric acid



which should contain luteinizing activity were more active in this respect than the extracts that underwent tryptic digestion.

One may thus postulate that in male animals the pituitary luteinizing gonadotrophin stimulates the Leydig cells to secrete androgen which in turn inhibits the liberation of further gonadotrophin while stimulating the germinal epithelium of the testis. As the concentration of androgen diminishes so the secretion of pituitary gonadotrophin is restored and so on.

In the female birds injections of testosterone propionate promoted a growth of follicles where oestrogen had apparently no effect. Bullough [1943] reported that a high local concentration of oestrogen in the mouse ovary caused proliferation of the germinal epithelium, while large doses injected into the general circulation inhibit the hypophysis and cause follicular regression. Bullough suggests that the pituitary initially stimulates ovarian activity, and as soon as secretory function is established ovarian activity is self-regulated: Bullough did not use hypophysectomized mice to test this hypothesis, though Pencharz [1940] and Williams [1940, 1944] have demonstrated ovarian stimulation by oestrogens in hypophysectomized rats. The latter author described an increase in the number of medium-sized follicles in the ovary without any increase in their maximum size. We have not counted the number of follicles before and after oestrogen treatment, but the maturation of the follicles was certainly not affected. As far as the action of androgen is concerned we cannot be certain that it affects oogenesis, but the follicular growth observed indicates that androgen possibly shares in the functional regulation of the ovary. That the stimulation produced by androgens may also be observed in immature pigeons suggests that the gonadotrophin secretion in females is at a lower level than in males.

#### SUMMARY

Spermatogenesis may be restored in the atrophic testes of hypophysectomized pigeons by androgen treatment, and the same treatment may produce follicular growth in the atrophic ovaries of hypophysectomized female pigeons. Large doses of androgen given to adult pigeons during the non-breeding season or to immature male pigeons did not stimulate the testes but did stimulate the ovarian follicles of immature female pigeons.

The roles of androgens in maintaining testicular function and in relation to pituitary function are discussed.

We are very grateful to Dr I. T. Hsu, of the Department of Anatomy, West China Union University, for his help in preparing the histological sections; to Prof. Chiao Tsai for reading the manuscript; to Organon Laboratories for supplies of testosterone propionate and oestrone; and to Dr A. S. Parkes, of London, for the pituitary extracts AP118B and AP81B.

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